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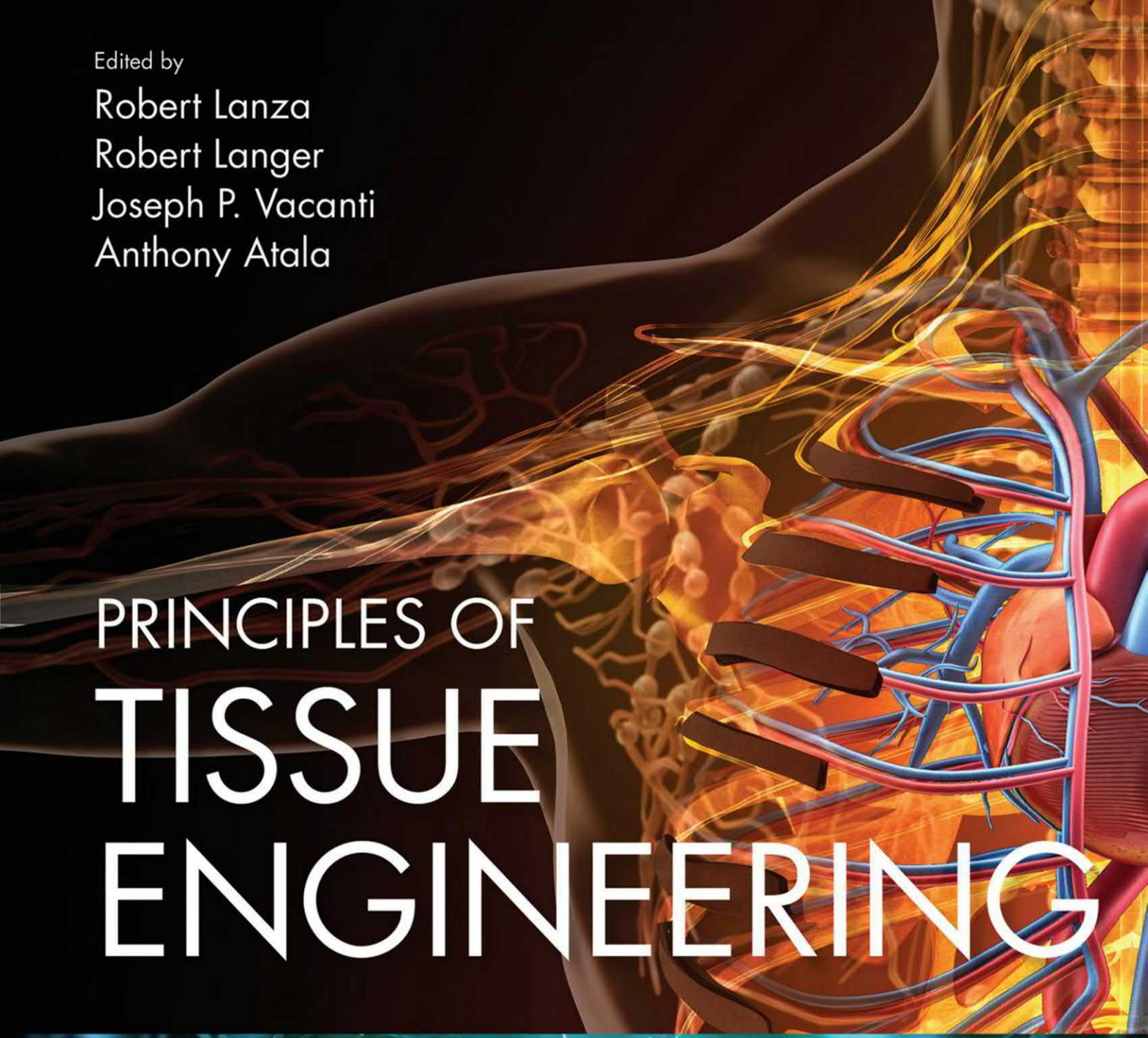

PRINCIPLES OF TISSUE ENGINEERING

FIFTH EDITION

Principles of Tissue Engineering

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Fifth Edition

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Contents

List of contributors xxix
Preface xli

1. Tissue engineering: current status and future perspectives 1

Prafulla K. Chandra, Shay Soker and Anthony Atala

Clinical need 1
Current state of the field 1
Smart biomaterials 2
Cell sources 4
Whole organ engineering 8
Biofabrication technologies 9
Electrospinning 9
Inkjet three-dimensional bioprinting 12
Extrusion three-dimensional bioprinting 12
Spheroids and organoids 13
Imaging technologies 16
Tissue neovascularization 16
Bioreactors 16
Organ-on-a-chip and body-on-a-chip 17
Integration of nanotechnology 18
Current challenges 19
Future directions 21
Smart biomaterials 21
Cell sources 22
Whole organ engineering 24
Biofabrication technologies 24
Tissue neovasculatization 25
Bioreactors 25
Integration of nanotechnology 25
Conclusions and future challenges 26
References 26
Further reading 35

2. From mathematical modeling and machine learning to clinical reality 37

Ben D. MacArthur, Patrick S. Stumpf and Richard O.C. Oreffo

Introduction 37
Modeling stem cell dynamics 37
Positive feedback–based molecular switches 38
Variability in stem cell populations 40
Modeling tissue growth and development 41
Monolayer tissue growth in vitro 42
Tissue growth on complex surfaces in vitro 42
Three-dimensional tissue growth in vitro 43
Pattern formation 44
Machine learning in tissue engineering 45
Supervised methods 46
Unsupervised methods 46
Machine learning of cellular dynamics 47
Regulatory network inference 47
From mathematical models to clinical reality 47
References 48

3. Moving into the clinic 53

Chi Lo, Darren Hickerson, James J. Yoo, Anthony Atala and Julie Allickson

Introduction 53
Current state of tissue engineering 53
Pathway for clinical translation 54
Regulatory considerations for tissue engineering 58
Conclusion 60
Acknowledgment 60
References 60
Further reading 60

Part One
The basis of growth and differentiation 63

4. Molecular biology of the cell 65

J.M.W. Slack

The cell nucleus 65
Control of gene expression 66
Transcription factors 67
Other controls of gene activity 67
The cytoplasm 68
The cytoskeleton 69
The cell surface 71

Cell adhesion molecules 71
Extracellular matrix 72
Signal transduction 73
Growth and death 74
Culture media 75
Cells in tissues and organs 76
Cell types 76
Tissues 77
Organs 77
Reference 78
Further reading 78

5. Molecular organization of cells 79

Jon D. Ahlstrom

Introduction 79
Molecules that organize cells 79
Changes in cell–cell adhesion 80
Changes in celleextracellular matrix adhesion 80
Changes in cell polarity and stimulation of cell motility 81
Invasion of the basal lamina 81
The epithelial–mesenchymal transition transcriptional program 82
Transcription factors that regulate epithelial–mesenchymal transition 82
Regulation at the promoter level 82
Posttranscriptional regulation of epithelial–mesenchymal transition transcription factors 83
Molecular control of the epithelial–mesenchymal transition 83
Ligand-receptor signaling 83
Additional signaling pathways 85
A model for epithelial–mesenchymal transition induction 85
Conclusion 86
List of acronyms and abbreviations 86
Glossary 86
References 87

6. The dynamics of cell–extracellular matrix interactions, with implications for tissue engineering 93

M. Petreaca and M. Martins-Green

Introduction 93
Historical background 93
Extracellular matrix composition 93
Receptors for extracellular matrix molecules 94
Cell–extracellular matrix interactions 96
Development 96
Wound healing 100
Signal transduction events during cell–extracellular matrix interactions 104
Relevance for tissue engineering 111
Avoiding a strong immune response that can cause chronic inflammation and/or rejection 111
Creating the proper substrate for cell survival and differentiation 111
Providing the appropriate environmental conditions for tissue maintenance 112
References 113

7. Matrix molecules and their ligands 119

Allison P. Drain and Valerie M. Weaver

Introduction 119
Collagens 120
Fibrillar collagens 121
Fibril-associated collagens with interrupted triple helices (FACIT) 122
Basement membrane–associated collagens 123
Other collagens 123
Major adhesive glycoproteins 123
Fibronectin 123
Laminin 125
Elastic fibers and microfibrils 126
Other adhesive glycoproteins and multifunctional matricellular proteins 126
Vitronectin 126
Thrombospondins 126
Tenascins 126
Proteoglycans 127
Hyaluronan and lecticans 127
Perlecan 128
Small leucine-rich repeat proteoglycans and syndecans 128
Conclusion 128
References 128

8. Morphogenesis and tissue engineering 133

Priscilla S. Briquez and Jeffrey A. Hubbell

Introduction to tissue morphogenesis 133
Biology of tissue morphogenesis 133
Morphogens as bioactive signaling molecules during morphogenesis 134
The extracellular matrix as a key regulator of tissue morphogenesis 135
Cell–cell interactions during tissue morphogenesis 136
Tissues as integrated systems in the body 136
Engineering tissue morphogenesis 138

Cells as building units in tissue engineering 138
Biomaterial scaffolds as artificial extracellular matrices 139
Morphogens as signaling cues in tissue engineering 140
Tissue remodeling in healthy and diseased environments 140
Current focuses and future challenges 141
References 141

9. Gene expression, cell determination, differentiation, and regeneration 145

Frank E. Stockdale

Introduction 145
Determination and differentiation 145
MyoD and the myogenic regulatory factors 147
Negative regulators of development 148
MicroRNAs—regulators of differentiation 148
Pax in development 149
Satellite cells in skeletal muscle differentiation and repair 149
Tissue engineering—repairing muscle and fostering regeneration by controlling determination and differentiation 150
Conclusion 152
References 152

Part Two
In vitro control of tissue development 155

10. Engineering functional tissues: in vitro culture parameters 157

Jennifer J. Bara and Farshid Guilak

Introduction 157
Key concepts for engineering functional tissues 158
Fundamental parameters for engineering functional tissues 158
Fundamental criteria for engineering functional tissues 159
Importance of in vitro studies for engineering functional tissues 159
In vitro studies relevant to tissue engineering and regenerative medicine 159
In vitro platforms relevant for high throughput screening of drugs and other agents 160
Influence of selected in vitro culture parameters on the development and performance of engineered tissues 161
Culture duration 161
Biomaterials 162
Bioreactors and growth factors 166
Bioreactors and mechanical forces 169
Conclusion 171
Acknowledgments 172
References 172
Further reading 177

11. Principles of bioreactor design for tissue engineering 179

Hanry Yu, Seow Khoon Chong, Ammar Mansoor Hassanbhai, Yao Teng, Gowri Balachander, Padmalosini Muthukumaran, Feng Wen and Swee Hin Teoh

Introduction 179
Macrobioreactors 180
Design principles 181
Sustainable bioreactors 188
Cell manufacturing quality attributes and process analytics technology 189
Future outlook 189
Microbioreactors 191
Design principles 191
Types of microreactors 194
Components and integration into microreactors 194
Applications 195
Summary 197
Acknowledgments 197
References 197

12. Regulation of cell behavior by extracellular proteins 205

Amy D. Bradshaw

Introduction 205
Thrombospondin-1 205
Thrombospondin-2 207
Tenascin-C 208
Osteopontin 209
Secreted protein acidic and rich in cysteine 210
Conclusion 212
References 212

13. Cell and matrix dynamics in branching morphogenesis 217

Shaimar R. González Morales and Kenneth M. Yamada

Introduction 217
The basis of branching morphogenesis 217

Branching morphogenesis in the lung 218
Branching morphogenesis in the salivary gland 220
Branching morphogenesis in the kidney 222
Contributions of other cell types 224
MicroRNAs in branching morphogenesis 225
Extracellular matrix components in branching morphogenesis 226
Laminin 226
Collagen 226
Heparan sulfate proteoglycan 227
Fibronectin and integrins 228
Basement membrane microperforations 228
Mathematical and computational models 230
Geometry 230
Mechanical forces 230
Signaling mechanisms 230
Conclusion 231
Acknowledgments 232
References 232

14. Mechanobiology, tissue development, and tissue engineering 237

David Li and Yu-li Wang

Introduction 237
Mechanical forces in biological systems 237
Tension 237
Compression 238
Fluid shear 238
Cellular mechanosensing 238
The cytoskeleton 239
Stretch-activated ion channels 239
Cell–cell adhesions 240
Cell–substrate adhesions 240
The extracellular matrix 241
Cellular effects of mechanotransduction 243
Substrate adhesion, spreading, and migration 243
Cell–cell interactions in collectives 243
Proliferation and differentiation 244
Mechanotransduction in biological phenomena 245
Wound healing 245
Tissue morphogenesis 247
Cancer metastasis 248
Mechanobiology in tissue engineering 248
Bone-implant design 248
Organs-on-a-chip 250
References 252

Part Three
In Vivo Synthesis of Tissues and Organs 257

15. In vivo engineering of organs 259

V. Prasad Shastri

Introduction 259
Historical context 259
Nature's approach to cellular differentiation and organization 260
Conceptual framework of the in vivo bioreactor 261
In vivo bone engineering—the bone bioreactor 261
In vivo cartilage engineering 264
Induction of angiogenesis using biophysical cues—*organotypic vasculature engineering* 265
De novo liver engineering 267
Repairing brain tissue through controlled induction of reactive astrocytes 269
Conclusions and outlook 269
References 270

Part Four
Biomaterials in tissue engineering 273

16. Cell interactions with polymers 275

W. Mark Saltzman and Themis R. Kyriakides

Methods for characterizing cell interactions with polymers 275
In vitro cell culture methods 275
In vivo methods 278
Cell interactions with polymers 280
Protein adsorption to polymers 280
Effect of polymer chemistry on cell behavior 280
Electrically charged or electrically conducting polymers 284
Influence of surface morphology on cell behavior 284
Use of patterned surfaces to control cell behavior 285
Cell interactions with polymers in suspension 286
Cell interactions with three-dimensional polymer scaffolds and gels 287
Cell interactions unique to the in vivo setting 287
Inflammation 287
Fibrosis and angiogenesis 288
References 289

17. Polymer scaffold fabrication 295

Matthew L. Bedell, Jason L. Guo, Virginia Y. Xie, Adam M. Navara and Antonios G. Mikos

Introduction 295
Design inputs: materials, processing, and cell types 297
Materials and inks 297
Processing and cell viability 299
Cell types and biological interactions 300
Assessment of cell viability and activity 301
3D printing systems and printer types 302
Inkjet printing 303
Extrusion printing 304
Laser-assisted bioprinting 305
Stereolithography 305
Open source and commercial 3D printing systems 306
Print outputs: patterning, resolution, and porous architecture 307
Printing/patterning of multiple inks 308
Print resolution 308
Porous architecture 309
Assessment of scaffold fidelity 309
Printing applications: vascularized and complex, heterogeneous tissues 310
Conclusion 310
Acknowledgments 311
Abbreviations 311
References 311

18. Biodegradable polymers 317

Julian Chesterman, Zheng Zhang, Ophir Ortiz, Ritu Goyal and Joachim Kohn

Introduction 317
Biodegradable polymer selection criteria 317
Biologically derived polymers 318
Peptides and proteins 318
Biomimetic materials 322
Polysaccharides 322
Polyhydroxyalkanoates 325
Polynucleotides 326
Synthetic polymers 326
Aliphatic polyesters 326
Aliphatic polycarbonates 330
Biodegradable polyurethanes 330
Polyanhydrides 331
Polyphosphazenes 331
Poly(amino acids) and pseudo-poly (amino acids) 332
Combinations (hybrids) of synthetic and biologically derived polymers 333
Using polymers to create tissue-engineered products 333
Barriers: membranes and tubes 334
Gels 334
Matrices 334
Conclusion 335
References 335

19. Three-dimensional scaffolds 343

Ying Luo

Introduction 343
Three-dimensional scaffold design and engineering 343
Mass transport and pore architectures 344
Mechanics 346
Electrical conductivity 348
Surface properties 349
Temporal control 352
Spatial control 354
Conclusion 355
References 355

Part Five
Transplantation of engineered cells and tissues 361

20. Targeting the host immune response for tissue engineering and regenerative medicine applications 363

Jenna L. Dziki and Stephen F Badylak

Introduction 363
Immune cells and their roles in building tissues after injury 363
Neutrophils 364
Eosinophils 364
Macrophages 364
Dendritic cells 364
T and B cells 365
Specialized immune cell functions beyond host defense 365
Tissue engineering/regenerative medicine strategies as immunotherapy 365
Future considerations for immune cell targeting tissue engineering/regenerative medicine therapies 366
References 366
Further reading 368

21. Tissue engineering and transplantation in the fetus 369

Christopher D. Porada, Anthony Atala and Graça Almeida-Porada

Introduction 369
Rationale for in utero therapies 370
In utero transplantation 371
Early murine experiments with in utero transplantation 372
In utero transplantation experiments in large preclinical animal models 372
Barriers to in utero transplantation success 373
Clinical experience with in utero transplantation 376
Rationale for in utero gene therapy 376
Hemophilia A as a model genetic disease for correction by in utero gene therapy 377
The need for better hemophilia A treatments 378
Preclinical animal models for hemophilia A and recent clinical successes 378
Sheep as a preclinical model of hemophilia A 379
Feasibility and justification for treating hemophilia A prior to birth 380
Mesenchymal stromal cells as hemophilia A therapeutics 383
Preclinical success with mesenchymal stromal cell–based hemophilia A treatment 384
Risks of in utero gene therapy 385
Genomic integration–associated insertional mutagenesis 385
Potential risk to fetal germline 386
Conclusion and future directions 387
References 388

22. Challenges in the development of immunoisolation devices 403

Matthew A. Bochenek, Derfogail Delcassian and Daniel G. Anderson

Introduction 403
Rejection and protection of transplanted cells and materials 403
Rejection pathways 404
Cellular nutrition 404
Therapeutic cells 405
Primary cells 405
Immortalized cell lines 406
Stem cells 407
Device architecture and mass transport 407
Transplantation site 408
Improving oxygenation of immunoprotected cells 409
Controlling immune responses to implanted materials 410
Steps in the foreign body reaction 411
The role of geometry in the foreign body reaction 411
Tuning chemical composition to prevent attachment 412
Directing immune cell behavior in the transplant niche 412
References 412

Part Six
Stem cells 419

23. Embryonic stem cells 421

Irina Klimanskaya, Erin A. Kimbrel and Robert Lanza

Introduction 421
Approaches to human embryonic stem cell derivation 421
Maintenance of human embryonic stem cell 425
Subculture of human embryonic stem cell 425
Nuances of human embryonic stem cell culture 426
Directed differentiation 426
Safety concerns 430
Conclusion 431
Acknowledgment 431
References 431

24. Induced pluripotent stem cell technology: venturing into the second decade 435

Yanhong Shi, Haruhisa Inoue, Jun Takahashi and Shinya Yamanaka

Disease modeling 435
Drug discovery 436
Stem cell–based therapeutic development 438
Concluding remarks 440
Acknowledgements 440
References 440

25. Applications for stem cells 445

Andres M. Bratt-Leal, Ai Zhang, Yanling Wang and Jeanne F. Loring

Introduction 445
Reprogramming of somatic cells into induced pluripotent stem cells 445
Epigenetic remodeling 446
Reprogramming techniques 446

Induced transdifferentiation 448
Genomic stability 448
Applications of induced pluripotent stem cells 448
Disease modeling 448
Challenges and future possibilities in disease modeling 450
Disease-modifying potential of induced pluripotent stem cells 451
Other applications for induced pluripotent stem cells 452
Conclusion 452
List of acronyms and abbreviations 453
References 453

26. Neonatal stem cells in tissue engineering 457

Joseph Davidson and Paolo De Coppi

Introduction 457
Stem cells 457
Embryonic stem cells 457
Induced pluripotent stem cells 458
Perinatal stem cells 458
Scaffolding specifics in fetal and neonatal tissue engineering 459
Synthetic materials 459
Natural materials 459
Relevance to prenatal therapy 460
Immunology 460
Physiology 460
Conditions of interest 461
Spina bifida 461
Gastroschisis 461
Congenital diaphragmatic hernia 461
Esophageal atresia 461
Congenital heart disease 462
Congenital airway anomalies 462
Bladder 463
Bone and bone marrow 463
Conclusion 463
References 463

27. Embryonic stem cells as a cell source for tissue engineering 467

Ali Khademhosseini, Nureddin Ashammakhi, Jeffrey M. Karp, Sharon Gerecht, Lino Ferreira, Nasim Annabi, Mohammad Ali Darabi, Dario Sirabella, Gordana Vunjak-Novakovic and Robert Langer

Introduction 467
Maintenance of embryonic stem cells 468
Directed differentiation 471
Genetic reprogramming 471
Microenvironmental cues 472
Three-dimensional versus two-dimensional cell culture systems 475
High-throughput assays for directing stem cell differentiation 475
Physical signals 477
Isolation of specific progenitor cells from embryonic stem cells 479
Transplantation 480
Transplantation and immune response 481
Future prospects 482
Conclusion 483
Acknowledgments 483
Conflicts of interest 483
References 483
Further reading 490

Part Seven
Gene therapy 491

28. Gene therapy 493

Stefan Worgall and Ronald G. Crystal

Strategies of gene therapy 493
Ex vivo versus in vivo gene therapy 494
Ex vivo 494
In vivo 495
Chromosomal versus extrachromosomal placement of the transferred gene 495
Gene transfer vectors 495
Nonviral vectors 497
Adenovirus 497
Adeno-associated virus 499
Retrovirus 500
Lentivirus 501
Cell-specific targeting strategies 502
Targeting of Ad vectors 502
Targeting of adeno-associated virus vectors 505
Targeting of retroviral and lentiviral vectors 505
Regulated expression of the transferred gene 505
Using gene transfer vectors for gene editing 507
Combining gene transfer with stem-cell strategies 508
Gene transfer to stem cells 508
Gene transfer to control uncontrolled stem-cell growth 508
Gene transfer to instruct stem-cell differentiation 508
Gene transfer to regulate gene expression 509
Challenges to gene therapy for tissue engineering 509
Acknowledgments 510
References 510

29. Gene delivery into cells and tissues 519

Christopher E. Nelson, Craig L. Duvall, Aleš Prokop, Charles A. Gersbach and Jeffrey M. Davidson

Introduction **519**
Fundamentals of gene delivery **519**
Biodistribution, targeting, uptake, and trafficking **521**
Tissue biodistribution/targeting 521
Cellular uptake and intracellular trafficking 523
Viral nucleic acid delivery **526**
Introduction to viral gene therapy 526
Types of viral vectors 527
Engineering viral vectors 528
Nonviral nucleic acid delivery **530**
Introduction to nonviral nucleic acid delivery 530
Oligonucleotide modifications 531
Conjugates 531
Synthetic polymers 531
Polymers derived from natural sources or monomers 534
Lipid-based delivery systems 536
Inorganic nanoparticles 537
High-throughput screening 537
Engineering tissues with gene delivery **538**
Introduction to engineering tissue with gene delivery 538
Viral delivery to engineer tissues 538
Nonviral delivery from scaffolds 540
Nucleic acid delivery for tissue engineering advances into the clinic 541
Future challenges 541
Outlook **542**
Acknowledgments **543**
References **543**

Part Eight
Breast **555**

30. Breast tissue engineering: implantation and three-dimensional tissue test system applications 557

Karen J.L. Burg and Timothy C. Burg

Introduction **557**
Breast anatomy and development **557**
Breast cancer diagnosis and treatments **558**
Breast reconstruction **558**
Synthetic implants 559
Tissue flaps 559
Cell transplants 559
Cellular scaffolds 560
Special considerations 565
Breast cancer modeling **565**
Animal models 565
Breast tissue test systems 566
In silico breast cancer models **570**
Concluding remarks **571**
Acknowledgement **571**
References **571**

Part Nine
Cardiovascular system **577**

31. Cardiac progenitor cells, tissue homeostasis, and regeneration 579

Wayne Balkan, Simran Gidwani, Konstantinos Hatzistergos and Joshua M. Hare

Origin of cardiac stem/progenitor cells **579**
Modeling cardiac development with pluripotent stem cells **581**
In vivo fate mapping of cardiac progenitors **582**
Neonatal cardiac repair **582**
Reprogramming cardiac fibroblasts **584**
Cardiac resident mesenchymal stem cells 584
Cardiomyocytes and cardiac repair/regeneration **585**
Cell-based therapy **585**
Cardiac progenitor/stem cell therapy 586
Combination stem cell therapy 586
Pluripotent stem cells 586
Future directions **588**
References **588**

32. Cardiac tissue engineering 593

Yimu Zhao, George Eng, Benjamin W. Lee, Milica Radisic and Gordana Vunjak-Novakovic

Introduction **593**
Clinical problem **593**
Engineering cardiac tissue: design principles and key components **594**
Cell source 594
Scaffold 598
Biophysical stimulation 599
Directed cardiac differentiation of human stem cells **599**
Derivation of cardiomyocytes from human pluripotent stem cells 599
Purification and scalable production of stem cell–derived cardiomyocytes 601
Scaffolds **601**
Decellularization approach 601
Artificial scaffolds 602

Biophysical cues 604
Electrical stimulation 604
Mechanical stimulation 604
Perfusion 606
In vivo applications of cardiac tissue engineering 606
Engineered heart issue 606
Vascularized cardiac patches 608
Electrical coupling of cardiomyocytes on the heart 608
Modeling of disease 609
Generation of patient-specific cardiomyocytes 609
Engineered heart tissue models 609
Cardiac fibrosis 609
Titin mutation–related dilated cardiomyopathy 611
Diabetes-related cardiomyopathy 611
Chronic hypertension induced left ventricle hypertrophy 611
Barth syndrome 611
Tissue engineering as a platform for pharmacologic studies 611
Summary and challenges 612
Acknowledgments 612
References 612

33. Blood vessels 617

Luke Brewster, Eric M. Brey and Howard P. Greisler

Introduction 617
Normal and pathologic composition of the vessel wall 617
Developmental biology cues important in vascular tissue engineering 618
Conduits 618
Arteries 618
Veins 618
Current status of grafts in patients 618
Conduit patency and failure 618
Venous reconstruction 619
Hemodialysis vascular access 619
Inflammation and the host response to interventions and grafts 620
Host environment and the critical role of the endothelium 621
Prevalent grafts in clinical use 622
Vascular tissue engineering 623
Early efforts—in vitro tissue-engineered vascular grafts 623
Endothelial cell seeding 623
In vitro approaches to tissue-engineered vascular grafts 624
In vivo tissue-engineered vascular grafts 625
Bioresorbable grafts 625
The living bioreactor 626
Cellular and molecular mediators of graft outcome 626
Conclusion and predictions for the future 630
References 630

34. Heart valve tissue engineering 635

Kevin M. Blum, Jason Zakko, Peter Fong, Mark W. Maxfield, Muriel A. Cleary and Christopher K. Breuer

Introduction 635
Heart valve function and structure 635
Cellular biology of the heart valve 636
Heart valve dysfunction and valvular repair and remodeling 637
Heart valve replacement 638
The application of tissue engineering toward the construction of a replacement heart valve 640
Tissue engineering theory 640
Biomaterials and scaffolds 640
The search for appropriate cell sources 643
Cell seeding techniques 644
Bioreactors 645
Neotissue development in tissue engineered heart valves 645
Clinical applications of the tissue engineered heart valve 647
Conclusion and future directions 648
References 649

Part Ten
Endocrinology and metabolism 655

35. Generation of pancreatic islets from stem cells 657

Bárbara Soria-Juan, Javier López-Beas, Bernat Soria and Abdelkrim Hmadcha

Introduction 657
State-of-the-art 657
The challenge of making a β-cell 658
Recent achievements (first generation of pancreatic progenitors used in the clinic) 658
Need of late maturation: cabimer protocol 659
Strategies to maintain cell viability 659
Encapsulation and tolerogenic strategies 661
The concept of cellular medicament 661
Conclusion 662
Acknowledgments 662
References 662

36. Bioartificial pancreas: challenges and progress 665

Paul de Vos

Introduction 665
History of the bioartificial pancreas 666
Replenishable cell sources and encapsulation 666
Macro- or microedevices 667
Factors contributing to biocompatibility of encapsulation systems 669
Avoiding pathogen-associated molecular patterns in polymers 670
Natural and synthetic polymers 670
Multilayer capsule approaches 670
Antibiofouling approaches 671
Formation of polymer brushes 671
Immunomodulatory materials 672
Intracapsular environment and longevity of the encapsulated islet graft 672
Concluding remarks and future considerations 673
Acknowledgments 674
References 674

37. Thymus and parathyroid organogenesis 681

Craig Scott Nowell, Kathy E. O'Neill, Paul Rouse, Timothy Henderson, Ellen Rothman Richie, Nancy Ruth Manley and Catherine Clare Blackburn

Structure and morphology of the thymus 681
Thymic epithelial cells 682
Complexity of the thymic epithelium compartment 682
Functional diversity 683
In vitro T cell differentiation 683
Thymus organogenesis 685
Cellular regulation of early thymus organogenesis 685
Origin of thymic epithelial cells 686
Thymic epithelial progenitor cells 686
Human thymus development 688
Cervical thymus in mouse and human 688
Molecular regulation of thymus and parathyroid organogenesis 689
Molecular control of early organogenesis 689
Transcription factors and regulation of third pharyngeal pouch outgrowth 691
Specification of the thymus and parathyroid 692
Foxn1 and regulation of thymic epithelial cell differentiation 695
Medullary development and expansion 696
Maintenance and regeneration of thymic epithelial cells: Progenitor/stem cells in the adult thymus 696
Strategies for thymus reconstitution 697
Summary 698
Acknowledgments 699
References 699

Part Eleven Gastrointestinal system 707

38. Stem and progenitor cells of the gastrointestinal tract: applications for tissue engineering the intestine 709

Kathryn M. Maselli, Christopher R. Schlieve, Mark R. Frey and Tracy C. Grikscheit

Introduction 709
Stem cells of the intestine 709
Cell types of the epithelial layer 709
Stem and progenitor cell types 710
Signaling pathways in the intestinal epithelium 712
The Wnt pathway 712
The Notch pathway 713
Epidermal growth factor receptor/ErbB signaling 713
The Hedgehog pathway 714
The BMP pathway 714
Tissue engineering the intestine with stem/progenitor cells 714
Organ-specific stem cell progenitors versus pluripotent stem cells 714
Synthetic and biological scaffolds 715
Primary intestinal-derived organoid units 716
Pluripotent stem cell approaches—human intestinal organoids 717
Remaining barriers to the generation of tissue-engineered intestine 718
Conclusion 718
Acknowledgment 718
References 718

39. Liver stem cells 723

Dagmara Szkolnicka and David C. Hay

Introduction 723
Liver architecture and function 723
Liver development 723
Fetal liver stem cells 724
Hepatocytes and liver progenitors in organ regeneration 724

Molecular signaling and processes involved in liver regeneration 724
Hepatocytes' role in liver regeneration 725
Cholangiocytes and liver stem cells in liver regeneration 725
Pluripotent stem cell–derived hepatoblasts and hepatocytes 726
3D liver organoids and expansion 727
Pluripotent stem cell–derived liver organoids 728
Bile duct–derived organoids 728
Hepatocyte-derived organoids 728
Novel scaffolds for liver organoids 729
Organoids as a model to study liver cancer disease 730
Reprogramming of human hepatocytes to liver progenitors using different culture conditions 730
Conclusion 731
References 731
Further reading 736

40. Hepatic tissue engineering 737

Amanda X. Chen, Arnav Chhabra, Heather E. Fleming and Sangeeta N. Bhatia

Liver disease burden 737
Current state of liver therapies 738
Extracorporeal liver support devices 738
Biopharmaceuticals 738
Liver transplantation 738
Hepatocyte transplantation 740
Current clinical trials 740
In vitro models 740
Two-dimensional liver culture 741
Three-dimensional liver constructs 741
Physiological microfluidic models of liver 742
Controlling three-dimensional architecture and cellular organization 742
In vivo models 743
Cell sourcing 743
Cell number requirements 743
Immortalized cell lines 744
Primary cells 744
Fetal and adult progenitors 744
Reprogrammed hepatocytes 744
Extracellular matrix for cell therapies 744
Natural scaffold chemistry and modifications 745
Synthetic scaffold chemistry 745
Modifications in scaffold chemistry 745
Porosity 746
Vascular and biliary tissue engineering 746
Vascular engineering 746
Host integration 747
Biliary network engineering 747
Conclusion and outlook 747
References 748

Part Twelve
Hematopoietic system 755

41. Hematopoietic stem cells 757

Qiwei Wang, Yingli Han, Linheng Li and Pengxu Qian

Introduction 757
Hematopoietic stem cells and hematopoietic stem cells niche 757
Effects of biomaterials on hematopoietic stem cells 758
Applications 759
Engineering hematopoietic stem cells niche for in vitro expansion 759
Manipulation of the multilineage differentiation of hematopoietic stem cells 760
In vivo tracking hematopoietic stem cells 761
Future perspectives 761
Acknowledgments 761
References 761

42. Blood components from pluripotent stem cells 765

Erin A. Kimbrel and Robert Lanza

Introduction and history of modern hematology 765
Red blood cells 765
Megakaryocytes/platelets 769
White blood cells 770
Lymphocytes—T cells 770
Lymphocytes—NK cells 773
Lymphocytes—NKT cells 775
Monocyte-derived dendritic cells 776
Monocyte-derived macrophages 777
Granulocytes—neutrophils 778
Perspectives 779
References 779

43. Red blood cell substitutes 785

Andre Francis Palmer and Donald Andrew Belcher

Introduction 785
Replicating red blood cell functions 785
Hemoglobin-based oxygen carriers 785
Hemoglobin toxicity 787
Oxygen delivery 789

Viscosity and colloid osmotic pressure 789
Cross-linked and polymeric hemoglobin 790
Surface conjugated hemoglobin 790
Encapsulated hemoglobin 791
Sources of hemoglobin 791
Recombinant hemoglobin 792
Erythrocruorins 792
Perfluorocarbons 793
Perspectives 794
Organ transplant preservation 794
Cancer treatment 795
Tissue-engineered construct oxygenation 795
References 795

Part Thirteen
Kidney and genitourinary system 803

44. Stem cells in kidney development and regeneration 805

Kyle W. McCracken and Joseph V. Bonventre

Kidney development 805
Early embryonic origins of nephrogenic tissues 806
Development of the nephric duct and ureteric bud 808
Maintenance and differentiation of the nephron progenitor cell 809
Role of stromal lineages in kidney organogenesis 811
Nephron endowment 812
Kidney repair and regeneration 813
Stem cells in kidney repair 813
Sources of nephrogenic cells 814
Differentiation of renal tissue from pluripotent stem cells (organoids) 815
Conclusion 817
Disclosures 818
Acknowledgements 818
References 818

45. Tissue engineering of the kidney 825

Ji Hyun Kim, Anthony Atala and James J. Yoo

Introduction 825
Cell-based tissue engineering of the kidney 826
Cell sources 826
Tissue-engineered cellular three-dimensional renal constructs 830
Cell-free tissue engineering of the kidney 835
In situ kidney regeneration 835
Granulocyte-colony stimulating factor 835
Stromal cell–derived factor-1 837
Conclusion and future perspectives 837
Acknowledgment 838
References 838

46. Tissue engineering: bladder and urethra 845

Yuanyuan Zhang, James J. Yoo and Anthony Atala

Introduction 845
Cell sources 846
Bladder and ureter cells 846
Stem cell sources 846
Mechanism of cell therapy 848
Biodegradable biomaterials 850
Synthetic scaffolds 850
Natural collagen matrix 851
Preclinical models 854
Tissue regeneration models 854
Fibrotic bladder model 854
Clinical trials 856
Clinical translation 856
Clinical studies 857
Conclusion 858
References 858

47. Tissue engineering for female reproductive organs 863

Renata S. Magalhaes, James K. Williams and Anthony Atala

Introduction 863
Uterus 863
Acellular tissue engineering approaches for uterine tissue repair 864
Cell-seeded scaffolds for partial uterine repair 864
Scaffold-free approaches for partial uterine repair 865
Uterine cervix tissue engineering 865
Ovary 865
Tissue engineering ovarian follicles 866
Vagina 866
Tissue engineering approaches for neovagina reconstruction 866
Conclusion and future perspectives 867
References 867

48. Male reproductive organs 871

Hooman Sadri-Ardekani, John Jackson and Anthony Atala

Introduction 871
Testes 871

Spermatogonial stem cell technology 871
Androgen-replacement therapy 873
Ejaculatory system 874
Engineering vas deferens 874
Spinal ejaculation generator 875
Penis 875
Penile reconstruction 875
Penile transplantation 876
Stem cell therapy for erectile dysfunction 876
Conclusion 877
References 877

Part fourteen
Musculoskeletal system 881

49. Mesenchymal stem cells in musculoskeletal tissue engineering 883

Yangzi Jiang, Dan Wang, Anna Blocki and Rocky S. Tuan

Introduction 883
Mesenchymal stem cell biology relevant to musculoskeletal tissue engineering 883
Mesenchymal stem cell identification 883
Tissue sources of mesenchymal stem cells 885
Mesenchymal stem cell isolation and in vitro culture 886
Mesenchymal stem cell self-renewal and proliferation capacity 887
Skeletogenic differentiation of mesenchymal stem cells 888
Plasticity of mesenchymal stem cells 888
Mesenchymal stem cell heterogeneity 889
Mesenchymal stem cell effect on host immunobiology 889
Safety of using mesenchymal stem cells for transplantation 891
Mesenchymal stem cells in musculoskeletal tissue engineering 891
Cartilage tissue engineering 891
General properties of articular cartilage 892
Cells for cartilage tissue engineering 892
Bone tissue engineering 897
Osteochondral tissue engineering 898
Engineering other skeletal tissues with mesenchymal stem cells 899
Tendon/ligament 899
Meniscus 900
Gene therapy in musculoskeletal tissue engineering 901
Conclusion and future perspectives 901
Acknowledgments 902
References 902

50. Bone tissue engineering and bone regeneration 917

J.M. Kanczler, J.A. Wells, D.M.R. Gibbs, K.M. Marshall, D.K.O. Tang and Richard O.C. Oreffo

Introduction 917
Skeletal stem cells 917
Fracture repair—the (limited) self-reparative capacity of bone 919
A framework for bone repair: biomaterial-driven strategies for bone regeneration 922
Growth factors: biomimetic-driven strategies for bone regeneration 923
Bone biofabrication 924
Development of vascular bone 925
Preclinical development—ex vivo/in vivo small and large animal preclinical models 926
Clinical translation 929
Summary and future perspectives 931
Acknowledgments 931
References 931

51. Tissue engineering for regeneration and replacement of the intervertebral disk 937

Stephen R. Sloan Jr., Niloofar Farhang, Josh Stover, Jake Weston, Robby D. Bowles and Lawrence J. Bonassar

Introduction 937
Intervertebral disk structure and function 938
Cell-biomaterial constructs for intervertebral disk regeneration 940
Nucleus pulposus cell-biomaterial implants 940
Annulus fibrosus repair and regeneration 942
Composite cell-biomaterial intervertebral disk implants 944
Cellular engineering for intervertebral disk regeneration 945
Cell therapy preclinical studies 946
Cell therapy clinical studies 947
Growth factors and other biologics for intervertebral disk regeneration 948
In vitro studies 948
In vivo studies: growth factors 952
In vivo studies: other biologics 953
Gene therapy for intervertebral disk regeneration 953
Gene transfer studies: viral 954
Gene transfer studies: nonviral 954
Endogenous gene regulation 955
Gene therapy in summary 955

In vivo preclinical models for intervertebral disk regeneration and replacement 955
Concluding remarks 957
Acknowledgment 957
References 957

52. Articular cartilage injury 967

J.A. Martin, M. Coleman and J.A. Buckwalter

Introduction 967
Articular cartilage injury and joint degeneration 968
Mechanisms of articular cartilage injuries 968
Response of articular cartilage to injury 970
Matrix and cell injuries 970
Chondral injuries 971
Osteochondral injuries 971
Preventing joint degeneration following injury 972
Promoting articular surface repair 972
Penetration of subchondral bone 972
Periosteal and perichondrial grafts 973
Cell transplantation 973
Artificial matrices 973
Growth factors 973
Antiinflammatories 974
Conclusion 974
Acknowledgments 974
References 974
Further reading 977

53. Engineering cartilage and other structural tissues: principals of bone and cartilage reconstruction 979

Batzaya Byambaa and Joseph P. Vacanti

Introduction 979
Biomaterials for cartilage tissue engineering 979
Cell sources for cartilage tissue engineering 980
Biofabrication of cartilage tissue 981
Magnetic resonance imaging and computerized tomography scans 981
Scaffolds for cartilage tissue engineering 981
Bioprinting techniques for fabrication of cartilage constructs 982
Bioinks for cartilage tissue printing 982
Osteochondral tissue engineering 985
References 985

54. Tendon and ligament tissue engineering 989

Spencer P. Lake, Qian Liu, Malcolm Xing, Leanne E. Iannucci, Zhanwen Wang and Chunfeng Zhao

Introduction 989
Tendon and ligament composition, structure, and function 990
Composition 990
Structure 990
Function 990
Requirements for a tissue-engineered tendon/ligament 991
Scaffold 992
Cell 994
Bioactive factors 995
Three-dimensional bioprinting and bioink 996
Bioink inspired from ligament and tendon structures 997
Tissue engineering tendon and ligament in clinical application 998
Summary 999
References 1000

55. Skeletal tissue engineering 1007

Matthew P. Murphy, Mimi R. Borrelli, Daniel T. Montoro, Michael T. Longaker and Derrick C. Wan

Introduction 1007
Distraction osteogenesis 1008
Critical-sized defects 1010
Cellular therapy 1010
Cytokines 1013
Scaffolds 1014
Tissue engineering in practice 1016
Conclusion 1017
References 1017

Part Fifteen
Nervous system 1023

56. Brain implants 1025

Lars U. Wahlberg

Introduction 1025
Cell replacement implants 1025
Primary tissue implants 1025
Cell line implants 1027
Cell protection and regeneration implants 1028
Cell implants secreting endogenous factors 1028
Cell implants secreting engineered factors (ex vivo gene therapy) 1029
Encapsulated cell brain implants 1029
Controlled-release implants 1030
Combined replacement and regeneration implants 1030
Disease targets for brain implants 1031

Surgical considerations 1032
Conclusion 1032
References 1032

57. Brain–machine interfaces 1037

José del R. Millán and Serafeim Perdikis

Introduction 1037
Brain–machine interface signals 1037
Voluntary activity versus evoked potentials 1038
Mutual learning 1040
Context-aware brain–machine interface 1040
Future directions 1041
References 1042

58. Spinal cord injury 1047

Nicolas N. Madigan and Anthony J. Windebank

Introduction 1047
Epidemiology 1047
Spinal cord organization 1047
Spinal cord injury 1048
Available clinical interventions 1049
The continuum of physical, cellular, and molecular barriers to spinal cord regeneration 1049
The role of tissue engineering in spinal cord injury repair 1051
Bioengineering for integrated spinal cord biocompatibility 1052
Animal models of spinal cord injury 1052
Principles of biomaterial fabrication for spinal cord injury repair 1054
Biomaterials for spinal cord tissue engineering: natural polymers 1058
Extracellular matrix polymers 1058
Polymers from marine or insect life 1065
Polymers derived from the blood 1071
Biomaterials for spinal cord tissue engineering: synthetic polymers 1072
Poly α-hydroxy acid polymers 1073
Nonbiodegradable hydrogels 1077
Conclusion and future directions: the promise of clinical translation 1080
References 1080

59. Protection and repair of hearing 1093

Su-Hua Sha, Karl Grosh and Richard A. Altschuler

Introduction 1093
Protection from "acquired" sensory hair cell loss 1093
Oxidative stress and stress-related mitochondrial pathways 1094
Calcium influx 1094
Endoplasmic reticulum stress 1094
Prevention of ototoxicity 1094
Prevention of acoustic trauma 1096
Antiinflammatory agents 1097
Heat shock proteins 1097
Neurotrophic factors 1098
Protection from excitotoxicity: "acquired" loss of auditory nerve connections to hair cells 1098
Gene transfer for the prevention and treatment of genetic deafness 1099
Interventions for hair cell repair: gene therapy for transdifferentiation 1099
Interventions for repair: hair cell and auditory nerve replacement—exogenous stem cells 1101
Interventions for repair/replacement: cochlear prostheses 1101
Fully implantable cochlear prostheses 1101
Interventions for repair/replacement: central auditory prostheses 1102
Local delivery to cochlear fluids 1103
Conclusion 1103
Acknowledgments 1103
References 1104
Further reading 1112

Part Sixteen
Ophthalmic 1113

60. Stem cells in the eye 1115

Chao Huang, Julie Albon, Alexander Ljubimov and Maria B. Grant

Introduction 1115
Endogenous ocular stem cells 1115
Corneal stem cells 1115
Stromal stem cells 1119
Endothelial stem cells 1119
Conjunctival epithelial stem cells 1120
The bioengineered cornea 1120
Retinal progenitor cells 1120
Müller stem cells 1121
Retinal pigment epithelium stem cells 1121
Nonocular stem cells 1121
Induced pluripotent stem cells (iPSCs) 1121
Embryonic stem cells/iPSCs in retinal regeneration 1121
Bone marrow stem cells 1124
References 1126

61. Corneal replacement tissue 1135

Maria Mirotsou, Masashi Abe and Robert Lanza

Introduction 1135
Corneal anatomy and structure 1135
Epithelium 1136
Stroma 1138
Endothelium 1139
Conclusion 1140
References 1141

62. Retinal degeneration 1145

Erin A. Kimbrel and Robert Lanza

Epidemiology of visual impairment and blindness 1145
Structure/function of the retina and cell types affected in retinal degenerative diseases 1145
Age-related macular degeneration 1147
History of retinal pigment epithelium as a cellular therapy for age-related macular degeneration 1147
Retinal pigment epithelium from pluripotent stem cells 1149
Retinitis pigmentosa 1150
Photoreceptors from pluripotent stem cells 1151
Glaucoma 1153
Stem cell–based therapies to treat glaucoma 1154
Diabetic retinopathy 1155
Stem cell–based therapies to treat diabetic retinopathy 1155
Future directions and competing therapies 1156
References 1157

63. Vision enhancement systems 1163

Gislin Dagnelie, H. Christiaan Stronks and Michael P. Barry

Introduction 1163
Visual system, architecture, and (dys)function 1163
Current- and near-term approaches to vision restoration 1166
Enhancing the stimulus through optoelectronic and optical means 1166
Visual prostheses based on electrical tissue stimulation 1167
Retinal cell transplantation 1170
Optic nerve protection and regeneration 1171
Drug delivery 1172
Genetic interventions 1172
Emerging application areas for engineered cells and tissues 1173
Photosensitive structures 1174
Optogenetics 1174
Outer retinal cell transplantation 1177
Cell matrices supporting axonal regrowth 1177
Repopulating ischemic or diabetic retina 1178
Assessing the functional outcomes of novel retinal therapies 1178
Conclusion: toward 2020 vision 1179
Acknowledgment 1179
References 1179
Further reading 1183

Part Seventeen
Oral/Dental applications 1185

64. Biological tooth replacement and repair 1187

Anthony J. (Tony) Smith and Paul T. Sharpe

Introduction 1187
Tooth development 1187
Whole tooth-tissue engineering 1189
Stem cell-based tissue engineering of teeth 1189
Bioteeth from cell-seeded scaffolds 1189
Root formation 1190
Cell sources 1191
Dental-tissue regeneration 1191
Natural tissue regeneration 1191
Importance of the injury-regeneration balance 1192
Signaling events in dental regeneration 1193
Control of specificity of dental-tissue regeneration 1193
Dental postnatal stem cells 1194
Directed tissue regeneration 1195
Signaling-based strategies 1195
Cell- and gene-based strategies 1196
Conclusion 1197
References 1197

65. Tissue engineering in oral and maxillofacial surgery 1201

Simon Young, F. Kurtis Kasper, James Melville, Ryan Donahue, Kyriacos A. Athanasiou, Antonios G. Mikos and Mark Eu-Kien Wong

Introduction 1201
Special challenges in oral and maxillofacial reconstruction 1201
Current methods of oral and maxillofacial reconstruction 1204
Mandibular defects 1205
Maxillary defects 1207

Relevant strategies in oral and maxillofacial tissue engineering 1208
Bone applications 1208
Cartilage applications 1212
Oral mucosa applications 1214
Composite tissue applications 1215
Animal models 1215
The future of oral and maxillofacial tissue engineering 1216
References 1216

66. Periodontal tissue engineering and regeneration 1221

Xiao-Tao He, Rui-Xin Wu and Fa-Ming Chen

Introduction 1221
Stem cells for periodontal bioengineering 1222
Intraoral mysenchymal stem cells 1222
Periodontal tissue–derived stem cells 1223
Stem cells from apical papilla 1224
Dental follicle stem cells 1224
Hertwig's epithelial root sheath 1225
Stem cells from dental pulp or exfoliated deciduous teeth 1225
Extraoral mysenchymal stem cells 1225
Bone marrow–derived mysenchymal stem cells 1225
Adipose-derived stem cells 1226
Selection of cell types 1226
Signaling molecules 1227
Types of signals 1228
Crucial delivery barriers to progress 1230
Gene delivery as an alternative to growth factor delivery 1231
Scaffolding and biomaterials science 1232
Requirements of cell scaffolds 1232
Biomaterial-based immune modulation 1233
Classes of biomaterials 1233
Biomaterial redesign for periodontal application 1235
Periodontal bioengineering strategies 1236
Cell-free approaches 1237
Cell-based approaches 1239
Challenges and future directions 1242
Closing remarks 1243
Acknowledgments 1243
References 1243

Part Eighteen
Respiratory system 1251

67. Cell- and tissue-based therapies for lung disease 1253

Jeffrey A. Whitsett, William Zacharias, Daniel Swarr and Vladimir V. Kalinichenko

Introduction: challenges facing cell and tissue-based therapy for the treatment of lung disease 1253
Lung morphogenesis informs the process of regeneration 1254
Integration and refinement of signaling and transcriptional pathways during lung formation 1256
The mature lung consists of diverse epithelial and mesenchymal cell types 1256
Structure and function of pulmonary vasculature 1257
Embryonic development of alveolar capillaries 1258
Evidence supporting lung regeneration 1259
A diversity of lung epithelial progenitor/stem cells is active during regeneration 1260
Role of lung microvasculature in lung repair 1262
Endothelial progenitor cells in lung repair 1262
Pulmonary cell-replacement strategies for lung regeneration 1263
Induced pluripotent stem cells for study of treatment of pulmonary disease 1263
Differentiation of induced pluripotent stem and embryonic stem cells to pulmonary epithelial cell lineages 1264
Bioengineering of lung tissues 1265
Mesenchymal stromal cells and mesenchymal stromal cell products for the treatment of lung disease 1265
Important role of the extracellular matrix in lung structure and repair 1265
Tissue engineering for conducting airways 1266
Pulmonary macrophage transplantation for the treatment of interstitial lung disease 1266
Conclusion 1266
Acknowledgments 1266
References 1266

68. Lung tissue engineering 1273

Micha Sam Brickman Raredon, Yifan Yuan and Laura E. Niklason

Introduction 1273
Design criteria for pulmonary engineering 1273
Decellularized scaffolds and biofabrication approaches 1274
Pulmonary epithelial engineering 1276
Proximal airway engineering 1276
Distal airway engineering 1276
Mesenchymal support of pulmonary epithelium 1277
Pulmonary endothelial engineering 1277
Endothelial cell sources for lung tissue engineering 1278
Endothelial seeding into lung scaffolds 1278
Organomimetic endothelial culture 1279

Mesenchymal support of pulmonary microvasculature 1280
Bioreactor technologies for pulmonary engineering 1280
Conclusion 1281
References 1281

Part Nineteen
Skin 1287

69. Cutaneous epithelial stem cells 1289

Denise Gay, Maksim V. Plikus, Iris Lee, Elsa Treffeisen, Anne Wang and George Cotsarelis

Introduction 1289
Interfollicular epidermal stem cells 1289
Models for skin renewal: epidermal proliferative unit versus committed progenitor 1290
Hair follicle stem cells 1291
The bulge as stem cell source 1291
Defining characteristics of the bulge as a stem cell source 1292
Multiple hair follicle stem cell subpopulations by marker expression 1294
Stem cells of other ectodermal appendages 1295
Sebaceous glands 1295
Sweat glands 1296
Nails 1296
Hair follicle stem cells in skin homeostasis, wound healing, and hair regeneration 1297
Homeostasis 1297
Wound healing 1297
Wound-induced hair follicle neogenesis and regeneration 1298
Epithelial stem cells in aging 1298
Role of stem cells in alopecia 1299
Skin as an active immune organ 1300
Cross talk between hair follicles and the immune system 1300
The inflammatory memory of skin cells 1301
Tissue engineering with epidermal stem cells 1301
Epidermal stem cells as a therapy: the future 1302
Conclusion 1302
References 1302

70. Wound repair: basic biology to tissue engineering 1309

Richard A.F. Clark, Michael Musillo and Thomas Stransky

Introduction 1309
Basic biology of wound repair 1310
Inflammation 1310
Transition from inflammation to repair 1310
Reepithelialization 1310
Granulation tissue 1312
Wound contraction and extracellular matrix organization 1316
Chronic wounds 1317
Scarring 1318
Pathological scars 1318
Scarless healing 1319
Tissue engineered therapy with skin cells 1320
Engineered epidermal constructs 1320
Engineered dermal constructs 1321
Engineered skin substitutes 1321
Skin autograft harvesting without scarring 1322
Tissue-engineered therapy with stem cells, bioactives, and biomaterials 1322
References 1324

71. Bioengineered skin constructs 1331

Vincent Falanga

Introduction 1331
Skin structure and function 1331
The epidermis 1331
The dermis 1332
The process of wound healing 1333
Impaired healing and its mechanisms 1333
Acute versus chronic wound healing 1333
Bacterial colonization 1333
Growth factor imbalances 1334
Matrix metalloproteinase activity 1334
Moist wound healing in chronic wounds 1334
Ischemia 1334
Abnormalities at the cellular level 1335
Engineering skin tissue 1335
Design considerations 1335
Commercial considerations 1336
Process considerations 1337
Regulatory considerations 1337
Immunological considerations 1338
Summary: engineering skin tissue 1338
Epidermal regeneration 1338
Dermal replacement 1339
Bioengineered living skin equivalents 1339
Bioengineered skin: FDA-approved indications 1340
Cutaneous indications 1340
Oral indications 1341
Apligraf and Dermagraft: off-label uses 1341
The importance of wound bed preparation 1344
Proposed mechanisms of action of bioengineered skin 1345

Construct priming and a new didactic paradigm for constructs 1347
Other considerations 1348
Conclusion 1348
References 1349
Further reading 1352

Part Twenty
Tissue-engineered food 1353

72. **Principles of tissue engineering for food** 1355

Mark Post and Cor van der Weele

Introduction 1355
Why tissue engineering of food? 1355
Specifics of tissue engineering for medical application 1356
Uniqueness 1356
Function 1356
Skeletal muscle and fat tissue engineering 1357
Tissue engineering of skeletal muscle 1357
Tissue engineering of fat 1359
Specifics of food tissue engineering 1361
Scale 1361
Efficiency 1362
Taste, texture, juiciness 1362
Enhanced meat 1363
Other foods 1363
Consumer acceptance 1364
Regulatory pathway 1365
Conclusion 1365
References 1365

73. **Cultured meat—a humane meat production system** 1369

Zuhaib F. Bhat, Hina Bhat and Sunil Kumar

Introduction 1369
Need and advantages of cultured meat 1370
Cultured meat 1372
Scaffolding techniques 1372
Self-organizing tissue culture 1373
Organ printing 1375
Biophotonics 1375
Nanotechnology 1375
Challenges and requirements for industrial production 1375
Generation of suitable stem cell lines from farm-animal species 1376
Safe media for culturing of stem cells 1377
Safe differentiation media to produce muscle cells 1377
Tissue engineering of muscle fibers 1378
Scaffolds 1378
Industrial bioreactors 1379
Fields 1380
Atrophy and exercise 1380
Senescence 1381
Meat processing technology 1381
Associated dangers and risks 1381
Regulatory issues 1381
Consumer acceptance and perception 1382
Role of media in publicity of cultured meat 1382
Market for cultured meat 1382
Conclusion 1383
References 1384

Part Twentyone
Emerging technologies 1389

74. **Three-dimensional bioprinting for tissue engineering** 1391

Jun Tae Huh, James J. Yoo, Anthony Atala and Sang Jin Lee

Introduction 1391
3D Bioprinting strategy: from medical image to printed bioengineered tissue 1391
Three-dimensional bioprinting techniques 1392
Jetting-based bioprinting 1392
Extrusion-based bioprinting 1394
Laser-assisted bioprinting 1394
Laser-based stereolithography 1395
Digital light processing 1395
Hybrid and other techniques 1396
Biomaterials as bioinks for three-dimensional bioprinting 1396
Hydrogel-based bioinks for cell-based three-dimensional bioprinting 1396
Biodegradable synthetic polymers for structure-based three-dimensional bioprinting 1399
Scaffold-free cell printing 1399
Three-dimensional bioprinting in tissue engineering applications 1400
Three-dimensional bioprinted vascular structures 1400
In vitro tissue models 1400
Three-dimensional bioprinted implantable tissue constructs 1403
Conclusion and future perspectives 1409
Abbreviations 1410
Glossary 1410
References 1411

75. Biofabricated three-dimensional tissue models 1417

David B. Berry, Claire Yu and Shaochen Chen

Introduction 1417
Current methods of three-dimensional biofabrication 1418
Biomaterials for three-dimensional fabrication 1421
Three-dimensional tissue models for drug screening, disease modeling, therapeutics, and toxicology 1425
Conclusion and future directions 1435
Acknowledgments 1435
References 1435

76. Body-on-a-chip: three-dimensional engineered tissue models 1443

Thomas Shupe, Aleksander Skardal and Anthony Atala

Introduction 1443
Advanced in vitro modeling systems—progression from two-dimensional to three-dimensional models 1444
Organ-on-a-chip technologies and their applications 1445
Microengineering and biofabrication 1446
Liver-on-a-chip 1447
Vessel-on-a-chip 1447
Lung-on-a-chip 1448
Heart-on-a-chip 1448
Cancer-on-a-chip 1448
Body-on-a-chip: integrated multiorgan systems and future applications 1449
The importance of multiorganoid integration 1449
Cutting edge body-on-a-chip: the first highly functional multiorganoid systems 1452
Conclusion and perspectives 1455
References 1456

77. Monitoring and real-time control of tissue engineering systems 1459

Jean F. Welter and Harihara Baskaran

Introduction 1459
Current state-of-the-art 1460
General environmental monitoring and real-time control 1460
Tissue-level monitoring 1462
Mechanical properties 1462
Cell-level monitoring 1463
Reporter-based gene expression imaging 1463
Tissue-specific 1463
Cartilage monitoring and real-time control 1463
Skin 1464
Concluding remarks 1464
Acknowledgments 1465
References 1465

78. Biomanufacturing for regenerative medicine 1469

Joshua G. Hunsberger and Darren H.M. Hickerson

Current landscape of biomanufacturing 1469
Highlighting current workflows for biomanufacturing 1470
Current challenges in biomanufacturing for regenerative medicine 1470
Current platform technologies enabling biomanufacturing 1472
Regulatory challenges for biomanufacturing 1473
Food and Drug Administration guidance documents 1474
Creating standards 1475
The future: envisioned advanced biomanufacturing 1476
Closed-modular biomanufacturing systems 1476
Off-the-shelf products 1477
Preservation advances 1477
Synthetic biology advances 1477
Cell banking advances 1477
Medical applications for biomanufacturing in regenerative medicine 1477
Space exploration 1478
References 1479

Part Twentytwo Clinical experience 1481

79. Tissue-engineered skin products 1483

Jonathan Mansbridge

Introduction 1483
Types of therapeutic tissue-engineered skin products 1484
Components of tissue-engineered skin grafts as related to function 1484
Scaffold 1484
Keratinocytes 1485
Fibroblasts 1485
Extracellular matrix 1485
Subcutaneous fat 1485
Components of the immune system 1486
Melanocytes 1486
Adnexal structures 1487

Commercial production of tissue-engineered skin products 1487
Regulation 1487
Product development 1487
Overall concept 1487
Allogeneic cell source 1488
Viability of product and avoidance of a final sterile fill 1488
Shelf life 1488
Size, user convenience 1489
The manufacture of Dermagraft and TransCyte 1489
Cells 1489
Medium 1489
Bioreactor design 1490
The Dermagraft and TransCyte production processes 1490
Release specifications 1491
Distribution and cryopreservation 1491
Problems with commercial culture for tissue engineering 1492
Clinical trials 1492
Immunological properties of tissue-engineered skin 1493
Commercial success 1494
Mechanism of action 1494
Future developments 1495
Conclusion 1496
References 1496

80. Tissue-engineered cartilage products 1499

Henning Madry

Introduction 1499
Cartilage defects, osteoarthritis, and reconstructive surgical options 1499
Cartilage defects pathophysiology 1499
Surgical treatment options for articular cartilage defects 1500
Tissue-engineered cartilage products for orthopedic reconstruction 1500
Cells for tissue-engineered cartilage repair 1500
Scaffolds for clinical tissue-engineered cartilage repair 1501
Collagen scaffolds 1501
Hyaluronan 1502
Synthetic polymers 1502
Agarose and alginate 1502
Scaffold-free three-dimensional systems 1502
Bioreactors for tissue-engineered cartilage repair 1502
Clinical nomenclature of scaffold-based techniques 1503
Clinical generations of autologous chondrocyte implantation 1503
Acellular, scaffold-based products 1503
Particulated autologous or allogenic articular cartilage 1503
Commercial autologous chondrocyte implantation products 1503
MACI (Vericel, Cambridge, MA, United States) 1503
ChondroCelect (TiGenix, Leuven, Belgium) 1504
Spherox (Co.don, Berlin, Germany) 1504
Novocart 3D (Tetec, Reutlingen, Germany) 1504
BioSeed C (Biotissue, Geneva, Switzerland) 1504
Novocart Inject (Tetec, Reutlingen, Germany) 1504
Chondron (Sewon Cellontech, Seoul, Korea) 1505
Cartipatch (Tissue Bank of France, Génie Tissulaire, Lyon, France) 1505
CARTISTEM (Medipost, Seongnam, Korea) 1505
Clinical application of autologous chondrocyte implantation in reconstructive articular cartilage surgery 1505
Indications for autologous chondrocyte implantation 1505
Contraindications 1505
Surgical steps 1506
Clinical results of autologous chondrocyte implantation 1506
Overview 1506
Data from prospective randomized clinical trials 1507
Long-term results of autologous chondrocyte implantation 1508
Clinical factors affecting the clinical outcomes of autologous chondrocyte implantation 1508
Conflict of interest 1509
References 1509

81. Bone tissue engineering 1511

Hani A. Awad, Regis J. O'Keefe and Jeremy J. Mao

Introduction 1511
Conventional bone tissue engineering strategies: cells, scaffolds, and biofactors 1511
Delivery of molecules and/or scaffolds to augment endogenous bone regeneration 1512
Biomaterials development and three-dimensional printing 1513
Clinical successes and opportunities in regenerative repair of craniofacial defects 1516
Conclusion 1517
Acknowledgments 1517
References 1517

82. Tissue-engineered cardiovascular products 1521

Doris A. Taylor, Camila Hochman-Mendez, Joern Huelsmann, Abdelmotagaly Elgalad and Luiz C. Sampaio

Clinical situation/reality **1521**
Considerations for tissue-engineered cardiovascular constructs **1521**
Components for tissue-engineered cardiovascular constructs **1521**
Cell sources 1521
Scaffolds 1524
Tissue-engineered cardiovascular constructs **1525**
Vascular grafts 1525
Valves 1526
Cardiac patches 1527
Building the next level of complexity: whole heart 1529
Pathway to approval and commercialization **1530**
Future perspectives **1532**
References **1532**

83. Tissue organoid models and applications 1537

Timothy S. Leach, Anthony Dominijanni, Sean V. Murphy and Anthony Atala

Introduction **1537**
Cell sources **1537**
Types of organoid models **1538**
Cardiac organoid **1539**
Liver organoid **1540**
Brain organoid **1540**
Lung organoid **1541**
Gastrointestinal tract organoid **1541**
Other organoid models **1542**
Applications **1542**
Tumor and disease models 1542
Drug analysis 1543
Organ-on-a-chip 1544
Developmental biology 1544
Conclusion **1545**
References **1545**

Part Twenty three
Regulation, commercialization and ethics 1551

84. The regulatory process from concept to market 1553

Kyung Eun Sung, Judith Arcidiacono, Donald W. Fink Jr., Andrea Gray, Johnny Lam, Winson Tang, Iwen Wu and Raj K. Puri

Introduction **1553**
Regulatory background **1553**
Overview of development and approval process **1554**
Early-stage development **1554**
Chemistry, manufacturing, and controls 1555
Pharmacology and toxicology 1555
Clinical 1556
US Food and Drug Administration/sponsor meetings **1557**
Submitting an investigational new drug application **1557**
Required US Food and Drug Administration forms 1557
Investigational new drug application contents 1558
US Food and Drug Administration review of an original investigational new drug application submission 1559
Later-stage development topics **1559**
Compliance with current good manufacturing practice 1559
Product readiness for Phase 3 1559
Potency assay 1560
Pharmacology and toxicology 1560
Phase 3 clinical development 1560
Combination products **1561**
Tissue-engineered and regenerative medicine products **1562**
3D bio-printed tissue-engineered/regenerative-medicine products 1563
Medical devices **1563**
Least burdensome principles 1563
Breakthrough device program 1563
Evaluation of devices used with regenerative medicine advanced therapy 1564
Expedited review programs **1564**
Other regulatory topics **1565**
Minimal manipulation and homologous use of human cells, tissues, and cellular and tissue-based products 1565
Clinical research involving children 1566
Expanded access to investigational drugs for treatment use 1566
Charging for investigational drugs under an investigational new drug application 1566
Responsibilities of sponsors and investigators 1566
Clinical research conducted outside of the United States 1568
Use of standards 1568
US Food and Drug Administration international regulatory activities 1568
The role of cell-based products in medical product testing 1568
Conclusion **1568**
Acknowledgments **1568**

Appendix I: Code of Federal Regulations citations relevant to cellular product development 1569
Appendix II: The list of acronyms 1569
References 1570

85. Business issues 1573

Matthew Vincent

Introduction 1573
The aging population 1573
Rise of regenerative medicine 1575
Product development 1577
Embryonic stem cells 1578
Induced pluripotent stem cells 1579
Direct reprogramming of differentiated cells 1580
Small molecule-induced differentiation 1580
Reimbursement 1580
Conclusion 1582
References 1582

86. Ethical issues 1585

Laurie Zoloth

Introduction 1585
Duty and healing: natural makers in a broken world 1587
To make is to know: notes on an old problem about knowledge 1587
What is a thing? The perils of deconstruction 1588
What contextual factors should be taken into account, and do any of these prevent the development and use of the technology? 1588
What purposes, techniques, or applications would be permissible and under what circumstances? 1589
On what procedures and structures, involving what policies, should decisions on appropriate techniques and uses be based? 1590
Conclusion 1590
References 1590
Index 1593

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Preface

The first edition of *Principles of Tissue Engineering* was published almost a quarter-of-a-century ago—back in the 1990s when the term "tissue engineering" was first coined—and quickly became the most widely relevant and cited textbook in the field. Since that time there have been powerful developments, including breakthroughs at all stages of development, ranging from two Nobel Prizes for pioneering work in the area of stem cells, which could be used as an unlimited source of cells for repair and engineering of tissues and organs, to actual clinical therapies, ranging from skin and bladder replacement to cartilage, bone, and cardiovascular repair.

The fifth edition of "Principles" covers all of this tremendous progress as well as the latest advances in the biology and design of functional tissues and organs for repair and replacement, from mathematical models to clinical reality. We have also added Anthony Atala, the W.H. Boyce Professor and Director of the Wake Forest Institute for Regenerative Medicine, as a new editor and have expanded the book to include a new section on emerging technologies, including 3D bioprinting and biomanufacturing for tissue-engineering products. As in the previous editions, the book attempts to simultaneously connect the basic sciences with the potential application of tissue engineering to diseases affecting specific organ systems. While the fifth edition furnishes a much needed update of the rapid progress that has been achieved in the field in the last 6 years, we have retained the fundamentals of tissue engineering, as well as those facts and sections which, while not new, will assist scientists, clinicians, and students in understanding this exciting area of biology and medicine.

The fifth edition of "Principles" is divided into an introductory section, followed by 23 parts starting with the basic science of the field and moving upward into applications and clinical experience. The organization remains largely unchanged, combining the prerequisites for a general understanding of cellular differentiation and tissue growth and development, the tools and theoretical information needed to design tissues and organs, as well as a presentation by the world's experts of what is currently known about each specific organ system, including breast, endocrine and metabolism, ophthalmic, oral/dental applications, skin, and the cardiovascular, gastrointestinal, hematopoietic, kidney and genitourinary, musculoskeletal, nervous, and respiratory systems. We have again striven to create a comprehensive book that, on one hand, strikes a balance among the diversity of subjects that are related to tissue engineering, including biology, chemistry, material science, medicine, and engineering, while emphasizing those research areas that are likely to be of clinical value in the future.

While we cannot describe all of the new and updated material of the fifth edition, we continue to provide expanded coverage of stem cells, including neonatal, postnatal, embryonic, and induced pluripotent stem cells and progenitor populations that may soon lead to new tissue-engineering therapies for cardiovascular disease, diabetes, and a wide variety of other diseases that afflict humanity. This up-to-date coverage of stem cell biology and other emerging technologies is complemented by updated chapters on gene therapy, the regulatory process, and the challenges of tissue engineering for food and in vitro meat production, which someday may end up a routine part of our food system, potentially reducing environmental pollution and land use. As with previous editions, we believe the result is a comprehensive textbook that will be useful to students and experts alike.

Robert Lanza, Robert Langer, Joseph Vacanti and Anthony Atala

Chapter 1

Tissue engineering: current status and future perspectives

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Clinical need

Tissue and organ failure due to disease, injury, and developmental defects has become a major economical and healthcare concerns [1]. At present, use of donated tissues and organs is the clinical practice to address this situation. However, due to the shortage of organ donors, the increasing number of people on the transplant waiting lists, and an ever-increasing aging population, dependence on donated tissues and organs is not a practical approach. In addition, due to severe logistical constraints, many organs from donors cannot be matched, transported, and successfully transplanted into a patient within the very limited time available. In the United States alone, more than 113,000 people are on the National Transplant Waiting list and around 17,000 people have been waiting for more than 5 years for an organ transplant (US Department of Health and Human Services, Organ Procurement and Transplantation network; https://optn.transplant.hrsa.gov; data as of February, 2019). To address this critical medical need, tissue engineering (TE) has become a promising option. TE and regenerative medicine (RM) are multidisciplinary fields that combine knowledge and technologies from different fields such as biology, chemistry, engineering, medicine, pharmaceutical, and material science to develop therapies and products for repair or replacement of damaged tissues and organs [2,3].

The process of TE is multistep and involves engineering of different components that will be combined to generate the desired neo-tissue or organ (Fig. 1.1). Today, this field has advanced so much that it is being used to develop therapies for patients that have severe chronic disease affecting major organs such as the kidney, heart, and liver. For example, in the United States alone, around 5.7 million people are suffering from congestive heart failure [5], and around 17.9 million people die or cardiovascular diseases globally (World Health Organization data on Cardiovascular disease; https://www.who.int/cardiovascular_diseases/en/). TE can help such patients by providing healthy engineered tissues (and possibly whole organ in future) to replace their diseased tissue for restoring function. For example, chronic kidney disease (CKD) is a worldwide health crisis that can be treated, but it also depends on organ donation. In the United States alone, around 30 million people are suffering from CKD (Center for Disease Control & Prevention; National Chronic Kidney Disease Fact Sheet 2017; https://www.cdc.gov/kidneydisease/pdf/kidney factsheet), while close to 10% of the population is affected worldwide. Liver disease is another healthcare problem, which is responsible for approximately 2 million deaths per year worldwide [6]. Other diseases or conditions that can benefit from TE technologies include skin burns, bone defects, nervous system repair, craniofacial reconstruction, cornea replacement, volumetric muscle loss, cartilage repair, vascular disease, pulmonary disease, gastrointestinal tissue repair, genitourinary tissue repair, and cosmetic procedures. The field of TE, with its goal and promise of providing bioengineered, functional tissues, and organs for repair or replacement could transform clinical medicine in the coming years.

Current state of the field

TE has seen continuous evolution since the past two decades. It has also seen assimilating of knowledge and technical advancements from related fields such as material science, rapid prototyping, nanotechnology, cell biology, and developmental biology. Specific advancements that have benefited TE as a field in recent years include novel

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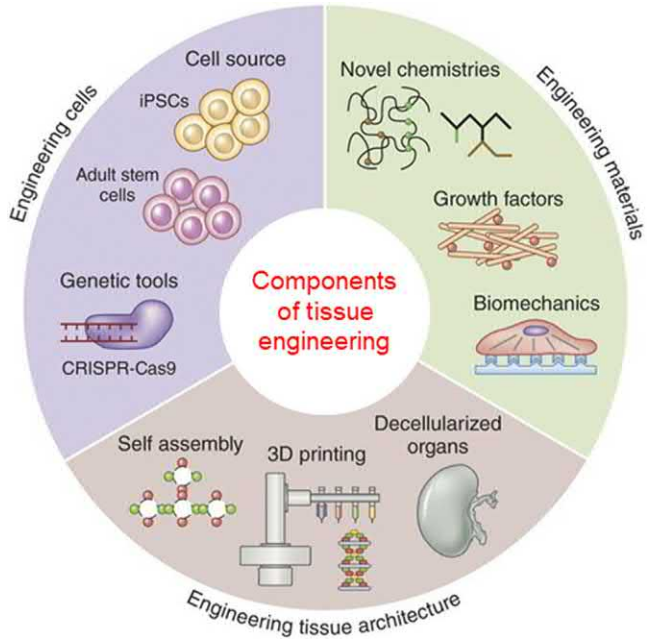

FIGURE 1.1 Schematic representation of different aspects of tissue engineering. Each component (materials, cells, and tissue architectures) can be engineered separately or in combination to achieve the therapeutic goals. *Reprinted with permission from Khademhosseini A., Langer R. A decade of progress in tissue engineering. Nat Protoc 2016;11 (10):1775–81. doi: 10.1038/nprot.2016.123 [4]. ©2016 Springer Nature Publishing AG.*

biomaterials [7], three-dimensional (3D) bioprinting technologies [8], integration of nanotechnology [9], stem-cell technologies such as induced pluripotent stem cells (iPSCs) [9,10], and gene editing technology such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) [11]. All these have led to promising developments in the field that include smart biomaterials, organoids, and 3D tissue for disease modeling and drug development, whole organ engineering, precise control and manipulation of cells and their environments, and personalized TE therapies.

Biomaterials are critical components of many current TE strategies. Recent developments in this field that are benefiting TE include synthesis of new biomaterials that can respond to their local environment and cues (smart biomaterials). Advancements in 3D bioprinting technologies are at the core of many developments in TE. It is now possible to print multiple biocompatible materials (both natural and synthetic), cells, and growth factors together into complex 3D tissues, many with functional vascular networks, which match their counterparts in vivo. We have also learned a great deal about cell sourcing, culture, expansion, and control of differentiation. This is also true for stem cells, where new sources such as placenta, amniotic fluid, and iPSCs have been explored and optimized for use. Vascularization and innervation in bioengineered tissue is a continuing challenge essential to warrant sustained efforts success of tissues implanted in vivo would be very low. Therefore there is a need for greater understanding of vascularization and innervation as applied to bioengineered tissues. This is an ongoing effort, and the results we are seeing from various studies are encouraging. Biofabrication technologies are playing a great role in this regards.

Several engineered tissues are moving toward clinical translation or are already being used in patients. These include cartilage, bone, skin, bladder, vascular grafts, cardiac tissues, etc. [12]. Although, complex tissues such as liver, lung, kidney, and heart have been recreated in the lab and are being tested in animals, their clinical translation still has many challenges to overcome. For in vitro use, miniature versions of tissues called organoids are being created and used for research in disease modeling, drug screening, and drug development. They are also being applied in a diagnostic format called organ-on-a-chip or body-on-a-chip, which can also be used for the above stated applications. Indeed, the development of 3D tissue models that closely resemble in vivo tissue structure and physiology are revolutionizing our understanding of diseases such as cancer and Alzheimer and can also accelerate development of new and improved therapies for multiple diseases and disorders. This approach is also expected to drastically reduce the number of animals that are currently being used for testing and research. In addition, 3D tissue models and organ-on-a-chip or body-on-a-chip platforms can support advancement of personalized medicine by offering patient-specific information on the effects of drugs, therapies, environmental factors, etc.

Development of advanced bioreactors represent another recent developments that are supporting clinical translation of TE technologies. Such bioreactors can better mimic in vivo environments by provide physical and biochemical control of regulatory signals to cells and tissue being cultured. Examples of such control include application of mechanical forces, control of electrical pacing, dynamic culture components, induction of cell differentiation. Incorporation of advanced sensors and imaging capabilities within these bioreactors are also allowing for real-time monitoring of culture parameters such as pH, oxygen consumption, cell proliferation, and factor secretion from a growing tissue. 3D modeling is also a new tool relevant to TE that provides great opportunities and better productivity for translational research, with wide clinical applicability [13]. Recent advancements in specific field that are helping advance TE are discussed next.

Smart biomaterials

Smart biomaterials are biomaterials that can be designed to modulate their physical, chemical, and mechanical

properties in response to changes in external stimuli or local physiological environment (Fig. 1.2) [14,15]. Advances in polymer synthesis, protein engineering, molecular self-assembly, and microfabrication technologies have made producing these next-generation biomaterials possible. These biomaterials can respond to a variety of physical, chemical, and biological cues such as temperature, sound, light, humidity, redox potential, pH, and enzyme activity [16,17]. Other unique characteristics displayed by some smart biomaterials are self-healing or shape-memory behavior [18]. The development of biomaterials with highly tunable properties has been driven by the desire to replicate the structure and function of extracellular matrix (ECM). Such materials can enable control of chemical and mechanical properties of the engineered tissue, including stiffness, porosity, cell attachment sites, and water uptake. For hydrogels, use of reversible cross-linking through physical methods, self-assembly, or thermally induced polymer chain entanglement is creating hydrogels that undergo structural changes in response to external stimuli [19,20]. Another class of hydrogels that are recent developments is called self-healing and shear thinning hydrogels. These materials are now being used to develop injectable biomaterials, which have low viscosity during application (injection) due to shear thinning and once at their target site, they self-crosslink (or heal) to fill the defect site [21]. Injectable biomaterials are also often loaded with drugs, biologics, and cells. For example, Montgomery et al. created an injectable shape-memory biomaterial for minimally invasive delivery of functional tissues [22]. In other applications, tissue glues are being developed using smart biomaterials, where they are used to bond and allow the tissue to self-heal. An example of this approach is a study by Bhagat and Becker

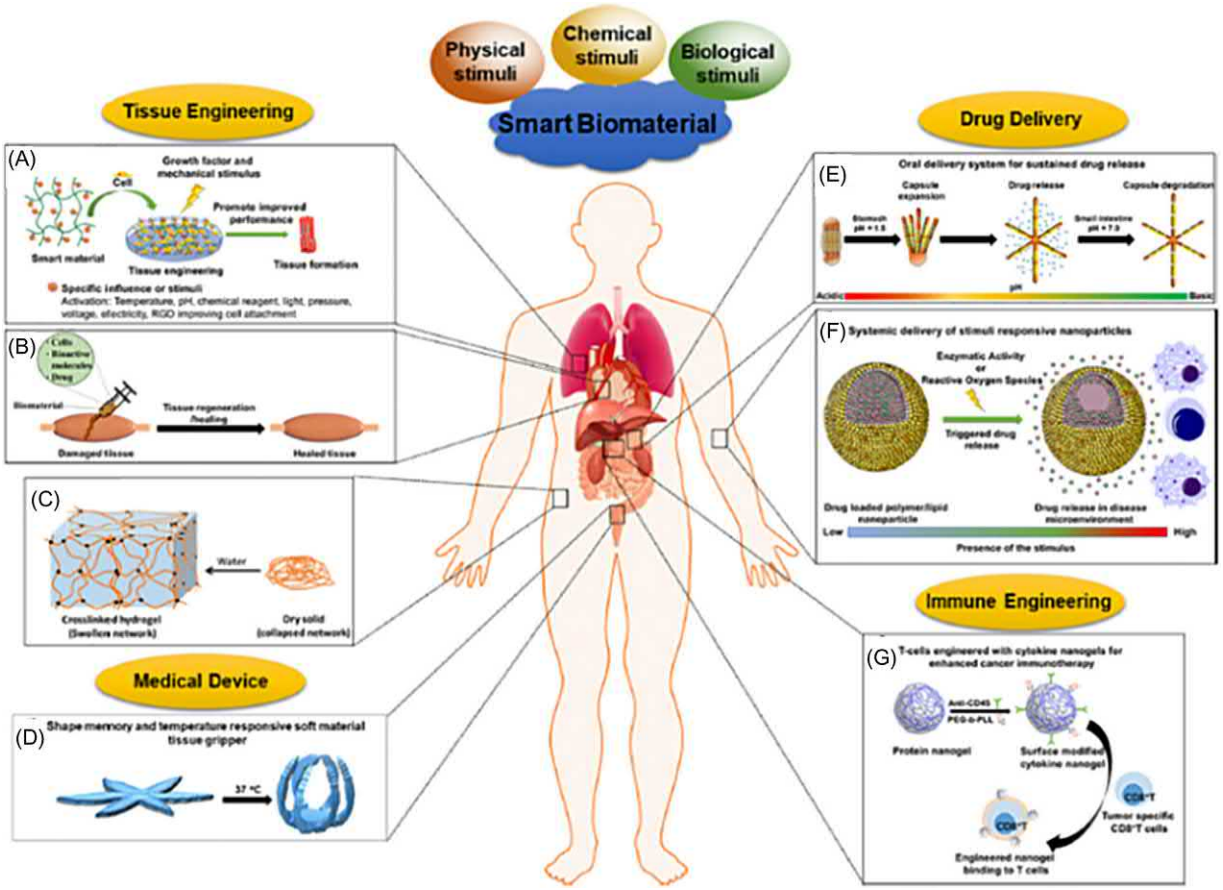

FIGURE 1.2 Different applications of smart biomaterials in the fields of tissue engineering and related fields. (A) Stimuli-responsive material that can promote cell differentiation and tissue growth; (B) injectable biomaterial loaded with cells, drugs, or bioactive molecules can be delivered less-invasively and can promote healing of tissue at the target damage site; (C) swelling polymer can be delivered as small scaffolds but can expand in vivo to achieve 3D structure of the target defect after exposure to water; (D) shape-memory and temperature-responsive soft material can be used as a tissue adhesive; (E) star-shaped delivery system for sustained drug release in the gastrointestinal tract; (F) nanoparticle-based stimuli-responsive drug delivery system for systemic application; (G) materials for enhanced cancer immunotherapy using targeted delivery of chimeric antigen receptor T cell. *3D*, Three-dimensional. *Reprinted with permission from Kowlaski PS, Bhattacharya C, Afewerki S, Langer R. Smart biomaterials: recent advances and future directions. ACS Biomater Sci Eng 2018;4(11):3809−17 [14].*

who created a chondroitin-based tissue glue that helps direct improved tissue repair [23].

The ECM is a complex and dynamic structural scaffold for cells within tissues and plays an important role in regulating cell function [1]. Given the role of the ECM in structural support of tissues, there has been significant effort in developing ECM-based scaffolds for TE and RM [24,25]. However, as with all materials implanted into the body, the immune response significantly influences the ability of scaffold-containing engineered tissues to integrate and functionally interact with the host [26]. Thus an emerging strategy in TE is to design materials that can directly control the host immune response [27]. For example, the Arg-Gly-Asp (RGD) of ECM proteins can exert immunomodulatory effects on both innate and adaptive immune cells while also having an inhibitory effect on phagocytosis and neutrophil chemotaxis [28]. In the context of TE, synthetic ECM-mimetic hydrogels containing the RGD sequence have been shown to cause increased cellular adhesion on polymer scaffolds and also have an antiinflammatory effects from macrophages [29,30]. Under certain conditions, the RGD peptides have also been found to effect cytokine secretion from T cells [31]. Therefore use of RGD as part of TE scaffolds or hydrogels can be used to enhance cells adhesion in addition to controlling the ability of macrophages to degrade and remodel the surrounding tissue environment.

Matrix metalloproteinases (MMPs) are a family of proteases that not only selective degrade a wide variety of ECM proteins but also interact with bioactive molecules, some of which have immunomodulatory effects [32,33]. So, another strategy to control the extent of matrix remodeling, integration of engineered tissues into native host tissues or invasion of immune cell into implanted materials could be by incorporating MMP-sensitive peptides into the TE constructs. Examples of this approach include studies by Patterson and Hubbell, who showed that the rate of scaffold material degradation depends on the MMP-sensitive peptide sequence, the type of MMP, and also the MMP concentrations [34]. In a separate study, West and Hubbell created biomimetic poly(ethylene glycol) (PEG) hydrogels that incorporated peptides that could be degraded by either a fibrinolytic protease (plasmin) or a fibroblast collagenase (MMP-1) [35,36]. One drawback of this using MMP-sensitive peptides in TE constructs is their immunogenicity and more work will be needed to get around this issue. Possibly, use of immunomodulatory domains along with MMP-sensitive peptides could support long-term viability and integration within native host tissues.

Another category of smart biomaterials is multidomain peptides (MDPs) hydrogels. These are injectable ECM mimetic materials that are engineered to form self-assembling meshes at the target site [37,38]. These MDPs can also control cellular behavior. For example, in a mouse study by Moore et al., MDPs alone were found to be biocompatible and had prohealing effects in vivo [39]. Hydrogel have also been prepared from multiple ECM mimetic peptides for the purpose of enhancing the viability of the biomaterial in vivo. Smart biomaterials are going to have a big impact on 3D printing of tissues and organs. By combining smart biomaterials with 3D bioprinting, a wide variety of architectures can be created which can further offer control over how these materials perform in a biological environments. Smart biomaterials can also be made from proteins. Some protein−protein interactions can be utilized to physically crosslink protein chains, while small coiled-coil domains within some proteins (called leucine zippers) can self-assemble into superhelical structures. Leucine zippers have been used to make hydrogels by physically crosslinking protein domains [40]. The stability of the leucine zipper self-assembly (and hence the hydrogel) can be controlled by changing the temperature. Another way to control the stability of some protein-based hydrogels is by arrangement of the interacting domains [41].

One drawback of hydrogels made of self-interacting protein domains is their low-to-moderate mechanical properties, which is not ideal for TE applications. However, these week interactions can be reinforced by introducing covalent bonds into the network (e.g., disulfide bonds between cysteine in the protein chains). This will not only improve the mechanical properties of the hydrogel but also its stability [42].

Cell sources

For TE, a variety of cell types are now being used. They include autologous, allogeneic, progenitors, adult unipotent or multipotent stem cells and iPSCs (Fig. 1.3). For some applications, the ability to expand a sufficient number of autologous cells from a small biopsy is well-established [44]. A good example is bladder augmentation, where smooth muscle and urothelium can be easily isolated from then native tissue, expanded in culture and used for engineering a new bladder tissue. However, in many cases, it is challenging to harvest and/or expand enough appropriate autologous cells for this purpose. Examples of such cell types include hepatocytes, kidney cells, insulin-producing pancreatic beta cells, cardiomyocytes, neurons. New sources or methods to obtain these cell types in quantities can advance engineering of these tissues/organs and significantly benefit treatment of associated diseases. Immature precursor cells present within tissue such as skin, cartilage, muscle, and bladder are essential for the expansion of corresponding cells from biopsies and enabling engineering of neo-tissues [45]. The extension of this approach to other tissue and organ

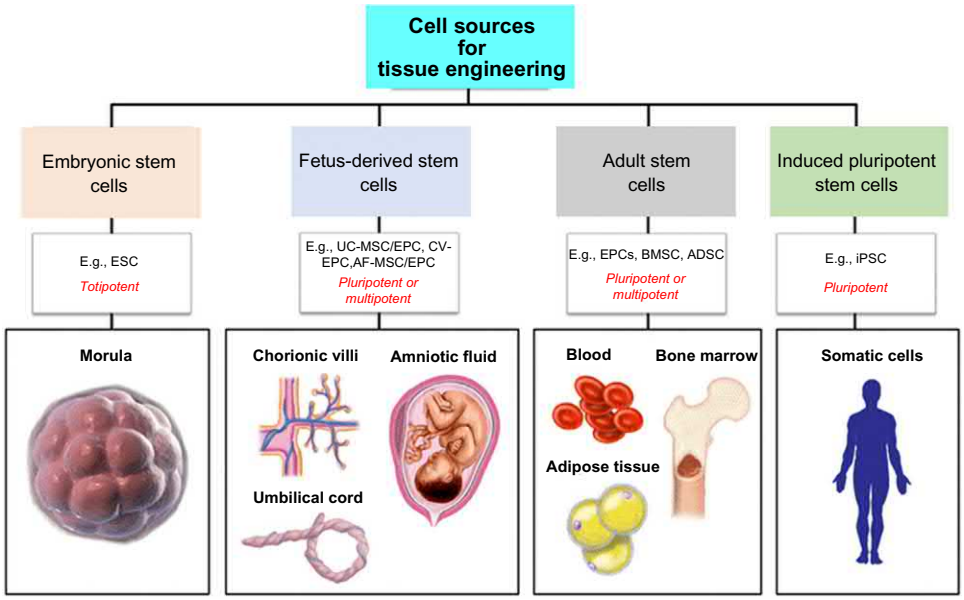

FIGURE 1.3 Different sources of cells for tissue engineering. Fetus-derived and induced pluripotent stem cells are gaining more attention for tissue engineering applications. *Reprinted from Al-Himdani S, Jessop ZM, Al-Sabah A, Combellack E, Ibrahim A, Doak SH, et al. Tissue-engineered solution in plastic and reconstructive surgery: principles and practice. Front Surg 2017;4:4. doi: 10.3389/fsurg.2017.00004. [43]. ©2017 Al-Himdani, Jessop, Al-Sabah, Combellack, Ibrahim, Doak, Hart, Archer, Thornton and Whitaker. Open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). Some portions of the original artwork have been modified.*

systems will depend greatly on finding sources of appropriate stem and progenitor cells.

Three major stem-cell sources are currently under intensive investigation:

1. embryonic stem (ES) cells, which are derived from discarded human embryos, and the equivalent embryonic germ (EG) cells;
2. iPSCs derived by genetic reprograming of somatic cells; and
3. Autologous or allogeneic adult tissue stem cells (sourced from fetal, neonatal, pediatric, or adult donor tissue).

Shared features of all stem cells include their capacity self-renewal and their ability to give rise to particular classes of differentiated cells. The ES, EG, and iPSCs can serve as precursors for many specialized cell type found during normal development and therefore are pluripotent. Adult stem cells are generally restricted to limited sets of cell lineages, hence called unipotent (constrained to a single fate) or multipotent (can give rise to multiple cell types). It appears likely that multiple tissue-engineered products based on each class of stem-cell source will be tested in the clinic in the coming years. Previous clinical and commercial experience sheds light on key differences between personalized products containing autologous cells and off-the-shelf products containing allogeneic cells. The vast majority of human studies till date have focused on using either adult stem or progenitor cells. More recently, clinical trials have begun with tissue-engineered products derived from pluripotent stem cells and their future looks promising.

The first clinical tissue-engineered products to achieve marketing approval from the US Food and Drug Administration (FDA) were skin substitutes that were used for wound healing. Examples of such products include Dermagraft (Shire Regenerative Medicine Inc., CT, United States) and Apligraf (Organogenesis, MA, United States), which were off-the-shelf products that used cells (fibroblasts for Dermagraft and fibroblasts plus keratinocytes for Apligraf) expanded from donated human foreskins. Whereas fibroblasts have been cultured in vitro since the early 20th century, the successful large-scale culture of human keratinocytes represented an important breakthrough for RM [46]. The success of off-the-shelf skin substitutes can be attributed to the lack of antigen-presenting cells, because of which they were not acutely rejected despite the inevitable histocompatibility mismatches between donors and recipients [47,48]. Eventually, the cells in the skin substitutes could be rejected, but the grafts has enough time for patients' own skin cells to regenerate. This stands in contrast to standard tissue/organ transplantation in which immune rejection is a major concern and immunosuppressive drug therapy is generally part of the application of allogeneic grafts [49]. Tissue-engineered products based on harvesting and expanding autologous cells containing stem and/or progenitor populations have also been developed successfully. Prominent examples include Epicel (Genzyme, MA, United States), a permanent skin replacement product based on expanded keratinocytes for patients with life-threatening burns, and Carticel (Genzyme, MA, United States), a chondrocyte-based treatment for large articular cartilage lesions [50,51].

Embryonic stem cells

ES cells and EG cells are indeed quite similar to early germ cells, with an apparently unlimited self-renewal capacity and pluripotency. Their great degree of plasticity represents both a strongest virtue and a significant potential limitation to their use in TE. A major ongoing challenge is in efficiently obtaining pure populations of specific desired specialized cell types from human ES cells [52,53]. Efforts during recent years have yielded more robust methods to isolate and grow ES cells under conditions consistent with Good Manufacturing Practice (GMP) and to generate differentiated cell products. While initial efforts have focused on cell therapies, these advances will positively impact production of tissue-engineered constructs using ES cells. Human ES cells are considerably more difficult to isolate and maintain stably in culture than the cell types that have previously been used in clinical testing. However, they can now be derived, grown, and cryopreserved without exposure to nonhuman cells or proteins, even under a GMP environment [54,55]. In the future, use of bioreactors, microcarriers, along with improved xeno-free and serum-free media and possibly small molecules that inhibit spontaneous differentiation of these cells would facilitate expansion of these stem cells to population sizes that are normally required for product development and clinical application [56,57].

Human tissues include more than 200 distinct cell types, and ES cells, in principle, can give rise to all of them. The historical approach of allowing ES cells to differentiate spontaneously has now been supplanted. Current strategies employ staged differentiation guided by knowledge of signaling events that regulate normal embryonic development [58]. For example, fine tuning of the exposure of early embryonic cells to the growth factor Nodal (a member of the transforming growth factor beta or TGF-β family) or its analog Activin A, in conjunction with other growth factors or small molecules, can now allow consistent generation of endoderm-specific cells from ES cells in vitro [59,60]. This is an early, but key milestone in a multistep process to generate differentiated cells that can eventually be used for TE of tissues/organs like the liver and pancreas. Conversely, inhibition of Nodal/Activin signaling favors the production of ectoderm specific cells, a precursor for neural lineage cells [61].

Despite substantial challenges, the first ES-cell-derived therapeutic product to enter clinical trials was the human ES-cell-derived oligodendrocyte progenitors (Geron Corporation; CA, United States) for stimulating nerve process growth in subjects with spinal cord injury [62]. Similarly, ES-cell-derived retinal pigment epithelium cells (Advanced Cell Technology, now Astellas Institute for Regenerative Medicine; CA, United States) were used in clinical trials in patients to treat Stargardt's macular dystrophy and dry age-related macular degeneration. Encouraging results from such clinical studies using ES cell-derived product will have a positive impact to develop tissue-engineered products from pluripotent stem cells in the near future. Areas of clear unmet medical need that might benefit from stem-cell-derived products include type 1 diabetes and Parkinson's disease. For type 1 diabetes, research at a biotech company called Viacyte Inc. (CA, United States) similarly pursued the produced progenitors of pancreatic endocrine cells from human ES cells using growth factors and hormones [63]. The progenitor cells from the final-stage differentiation in vitro were able to mature further in vivo to yield glucose-responsive β-like cells [64]. As a potential therapy for Parkinson's disease, significant advances have been made in the production of functional midbrain dopaminergic neurons by staged differentiation from ES cells [65,66]. Studies in the past few years have demonstrated that efficient grafting of these cells can lead to physiological correction of symptoms in several animal models, including nonhuman primates [67]. A particular safety concern is that undifferentiated pluripotent ES and iPS cells form teratomas in vivo. The risk of tumorigenicity makes it essential to rigorously determine the residual level of undifferentiated stem-cell population in any therapeutic product derived from ES or iPS cells [68]. It will also be valuable to determine whether a small number of undifferentiated pluripotent stem cells can be introduced into human patients without significant risk of tumor growth and if this threshold is influenced by use of immune suppressive drugs during treatment.

Induced pluripotent stem cells

Theoretically, the development of iPSCs represent the most direct way to ensure immune compatibility of tissue-engineered products when the recipient themselves serve as the donor. Generation of iPSCs through reprograming of mature somatic cells to a pluripotent state was first accomplished by ectopic expression of four transcription factors: OCT4 and SOX2, both with KLF4 and c-MYC [69] or NANOG and LIN28 [70]. The resulting iPSCs closely resembled ES cells in key properties such as the capacity for extensive self-renewal, ability to differentiate to multiple cell lineages, and generation of teratomas in vivo. Initial studies on reprograming of fibroblasts soon were extended to a variety of other cell types such as peripheral blood cells [71], cord blood cells [72], keratinocytes from hair shafts [73], and urine-derived cells [74]. Many recent developments have advanced this reprograming technology toward a safer, efficient translation toward therapeutic products. Also,

improved methods to deliver the pluripotency factors can minimize the risk of unintended permanent genetic modification of iPSCs, particularly integration of an oncogene such as c-*MYC* and thereby decrease the potential for future tumorigenicity [75]. One approach is being pursued for this is to transiently deliver the factors using various nonintegrating viral or plasmid vector systems. Reprograming also can be achieved by direct delivery of either synthetic messenger RNA (mRNA) encoding the pluripotency factors or of the protein factors themselves [76].

A recent development in the cellular reprograming field has centered on efforts to bypass the circuitous route of resetting cells to a pluripotent ground state and then inducing them to a desired lineage. Instead, there are efforts to achieve directed "trans-differentiation" between cell lineages. A number of studies have reported that fibroblasts or other adult cells can potentially be reprogramed directly to various specialized cell types such as neural progenitors [77], cardiomyocytes [78], endothelial cells [79], and hepatocytes [80]. However, there are still many queries about direct lineage-to-lineage reprogramming must be addressed. Some of these include the following questions: do the differentiated cells accurately mimic the genetic and functional characteristics of the target cells, or is there residual signatures of the original cells? Do the differentiated cells display fully adult phenotypes? Is the risk of introducing unwanted genetic or epigenetic abnormalities less or greater than in reprogramming through a pluripotent state? The answers to these questions will clarify the value of direct cell lineage conversions and the future of this approach for TE and RM applications.

Adult stem cells

Despite the promise of ES and iPSCs for TE and RM, the challenges of controlling lineage-specific differentiation, eliminating residual pluripotent stem cells, and confirming the safety and phenotype accuracy of then final products will likely delay the clinical translation and regulatory approval of such products. By contrast, adult stem cells represent a more straight-forward approach to rapid clinical development of cell-based and tissue-engineered products. Adult stem cells are present in many tissues throughout fetal development and postnatal life and are committed to restricted cell lineages [81,82]. Also, intrinsically they are not tumorigenic. At present, the most commonly used adult stem cells for development of cell therapy and TE applications are bone marrow-derived mesenchymal stromal or stem cells (MSC). MSCs can give rise to a number of tissue types, including cartilage, bone, adipose, and some types of muscle [83]. MSC have also generated considerable interest for musculoskeletal and vascular TE [84,85]. An advantage of using MSCs is that they can be easily harvested from liposuction specimens. An unexpected discovery that is further benefitting the use of MSCs for RM is then observation that they can be readily transplanted into allogeneic recipients without significant immune rejection [86]. This ability to avoid acute immune rejection in the host results from a variety of mechanisms, most notably the secretion of antiinflammatory cytokines [87]. Recent clinical trials have also assessed MSC-based cell therapy to treat graft versus host disease (GvHD) and various inflammatory or autoimmune conditions [88,89]. In fact, the first regulatory approvals for sale of a bone marrow-derived MSC product (Prochymal; Osiris Therapeutics; MD, United States) was for treatment of GvHD.

The therapeutic benefits of MSCs can also be through secretion of trophic factors. This has been seen in MSC-based cell therapy for heart disease, where delivery of autologous or allogeneic cells into the left ventricle wall for treatment of ischemic cardiomyopathy in a large animal model or even human clinical trials showed induction of new cardiomyocytes from endogenous cardiac stem and progenitor cells through trophic effects [90]. The injected MSCs also apparently contribute to positive remodeling of damaged heart tissue long after the initial damage [91]. Another study has demonstrated that combined delivery of MSCs with adult cardiac stem cells can substantially improves outcomes in the porcine model of ischemic cardiomyopathy [92]. This interesting result can be translated to generating improved tissue-engineered cardiac constructs by incorporating both the above stem cell types. Treatment of neurodegenerative conditions remains a challenge. Several years ago a company called Stem Cells Inc. (CA, United States) carried out clinical studies using a brain-derived neural stem-cell preparation called human central nervous system stem cells (HuCNS-SC), in a handful of subjects with neurological degenerative conditions referred to as neuronal lipoid fuscinosis (Batten's Disease) and Pelizaeus–Merzbacher disease [93]. Study data, including magnetic resonance imaging (MRI), demonstrated the durable engraftment of these cells and suggested that they contributed to myelination in recipient's brain tissue. The same company later began clinical trials using the same neural-derived stem cell in human subjects with dry age-related macular degeneration and spinal cord injuries.

Hepatic stem cells (HpSC) represent another human adult stem-cell population that can gives rise to parenchymal cells within tissues and organs [94]. The HpSCs are isolated from the liver and can be enriched from cadaveric fetal, neonatal, or fully mature donors by selection with a monoclonal antibody to the surface marker CD326. Exposure to certain growth factors (such as epidermal growth factor or EGF) or different tissue-specific matrix

molecules (such as liver proteoglycans) can induce efficient differentiation of the HpSC to either hepatocytes or cholangiocytes (bile duct cells) [95]. An early clinical study for the assessment of CD326-positive hepatic stem and progenitor cells on 25 subjects with decompensated liver cirrhosis found that delivery of these cells to the liver was best achieved by infusion via the hepatic artery. Also, at 6 months postinfusion, improvements in a number of clinical parameters were noted, including a significant decrease in the mean Mayo End-stage Liver Disease score ($P < .01$).

Another clinical study with HpSC transplantation achieved encouraging results using allogeneic donors, no human leukocyte antigen (HLA) loci matching, and without the use of immune suppressive drugs [94]. It is conceivable that HpSC (and possibly other fetal liver-derived stem cells) are particularly nonimmunogenic because they express only low levels of major histocompatibility complex (MHC) Class I and lack detectable MHC Class II (similar to ES cells). In addition, the liver is significantly immune privileged with respect to transplant rejection. Another hypothesis for this immune privilege could be that since this particular HpSC population were are isolated by immune-selection using antibody-coated beads specific for CD326, some angioblast-like mesenchymal cells could have copurified. Angioblast-like mesenchymal cells, just like MSC, are known to secrete immunomodulatory factors that could protect the HpSC and differentiated cells derived from them against immune rejection in the liver.

A new source of human adult stem-cell population, which can be used for engineering of pancreatic islet−like structures to treat insulin-dependent diabetes, was identified in peribiliary glands (found in the extrahepatic biliary tree located between the liver and pancreas) [95,96]. Molecular characterization of these biliary tree stem cells suggests that they comprise a population of endodermal stem cells that are more primitive than HpSC identified within the liver. Some of these biliary tree stem cells do not express CD326 but appear to be precursors of the CD326-positive HpSC. The biliary tree stem cells can proliferate extensively when cultured in the serum-free defined medium developed for HpSC.

Whole organ engineering

Tissue and organ failure is currently one of the biggest health issues, whose treatment is still an unmet medical need. This problem is ever increasing, with more than 100,000 patients being on the organ donor waiting lists in the United States alone [97]. Lack of sufficient organ donors and availability of healthy tissues and organs are further complicating this situation. TE is providing hope in this direction, with many efforts directed toward bioengineering tissues and even whole organs [98]. Decellularized tissues are gaining popularity as scaffolds for TE. These are prepared by removing cells from original tissues using mild detergents [99], after which they can be processed into different forms such as blocks, or powder for use. These decellularized materials represent the ECM of tissue from which they are derived and consist mainly of collagen. Since the shape, size, and complex structural properties of the native tissue are also maintained, decellularized tissue represent the ideal scaffolds for TE.

Decellularization can be performed using chemical, physical/ mechanical, or combination methods. Chemical methods include use of mild surfactants such as sodium dodecyl sulfate (SDS), sodium deoxycholate, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, Triton X-100, tridecylalcohol ethoxylate, and acid/bases such as per-acetic acid [100]. Physical/mechanical methods for decellularization are used in situations where there are concerns regarding the possible toxicity of the chemicals and nondesirable destruction of ECM proteins. Physical/mechanical treatments include the use of high hydrostatic pressure, freeze−thaw, or super-critical carbon dioxide (CO_2). The decellularization strategy is being used for TE to treat hernia repair [98,101], periodontal tissue [102], tendon [103], bone [104], vasculature [105], uterine tissue [106], heart valves [107], etc. Recent advancements in decellularized tissue research have resulted in successful decellularization of whole organs for whole organ reconstruction. Examples include liver [108], kidney [109], lung [110], and heart [111].

Clinical applications of decellularized ECM-based scaffolds are also on the rise. However, they have been limited to engineering of less complex tissues related to structural or reconstructive applications. It is noteworthy that many of the FDA-approved products on the market are derived from xenogeneic or allogeneic decellularized tissue ECM. Examples include SynerGraft for repair of human pulmonary heart valve (CryoLife; GA, United States), AlloDerm Regenerative Tissue Matrix (human dermal graft; LifeCell Corp, now Allergen, NJ, United States), and Meso BioMatrix Surgical Mesh (DSM Biomedical; PA, United States). Clinical trials have also been carried out using more complex structures made from decellularized ECM-scaffolds. One examples is a tissue-engineered trachea [112], which had long-term patency (at least 5 years posttransplantation), was completely cellularized and vascularized and did not provoke a significant immunogenic response [113].

Sometimes, the decellularization process can damage certain critical components needed for new tissue formation, such as endothelial basement membrane of the vasculature. In such cases, a practical strategy would be to add synthetic materials to promote functions. For

example, some researchers have used the immobilization of anticoagulants, such as peptides [114] or heparin [115], to the endothelium of the decellularized vessel to prevent blood coagulation inside the blood vessels during the regeneration process. A new avenue in decellularized tissue research is use of a device that links native tissues to synthetic materials. Here the decellularized tissue acts as an intermediate material, and then linking device ensures compatibility between the native tissue and synthetic materials at the molecular level. An example for this approach preparation of a decellularized skin dermis and poly(methyl methacrylate) (PMMA) complex by immersing the decellularized dermis in methyl methacrylate monomers, followed by polymerization [116]. Testing showed that this composite elasticity similar to the skin dermis, while the compressive modulus value was between that of the dermis and PMMA.

Biofabrication technologies

Biofabrication combines the principles of engineering, material science, and biology. It is a great toolbox that promise to change the outcome of many biomedical disciplines, particularly TE and RM. In addition, it also holds great potential for development of physiological 3D in vitro models, where complex tissue constructs are created that have a high degree of structural and functional similarities to native tissues. For TE, the most commonly used biofabrication technologies include (1) electrospinning; (2) drop-on-demand technologies such as ink-jet 3D bioprinting; (3) fused deposition modeling technologies such as extrusion-based 3D bioprinting; and (4) light-based technologies such as stereolithography (SLA) and laser-assisted bioprinting [117] (Fig. 1.4). Recent trends in electrospinning, inkjet printing, and extrusion-based 3D bioprinting are discussed next:

Electrospinning

Electrospinning refers to a technique for fabricating fibrous scaffolds [121] (Fig. 1.4B). The advantages of electrospinning as a scaffold fabrication technique include simple setup, versatility, and relative low-cost, which has supported use in TE applications, from skin grafts to vascular grafts to drug delivery devices [122–125]. For TE, a wide range of fiber architectures have been created, from scaffolds with uniform fibers to fibers with gradient properties, fibers with core–shell morphology, and scaffolds with patterned fiber depositions [126,127]. This has enabled researchers to create complex TE strategies to better mimic in vivo tissue structure and function. In spite of the several advantages of electrospun fibers and their scaffolds, one inherent limitation is the relatively poor cellular infiltration into the depth of these scaffold. This can happen due to high-fiber packing densities resulting in small and uneven pore sizes. Recent approaches in the field of electrospinning that have tried to address these limitations and expand the use of electrospun scaffolds in TE include use of postprocessing procedures and sacrificial components [128,129]. Some recent developments in electrospinning include modification of the electrospinning setup, new electrospinning processes, and new methods to achieve complex mesh composition and architectures (Table 1.1).

Modification of the electrospinning setup have been carried out to provide better fiber orientation, control of fiber blending or cospinning, and targeted fiber collection. Examples include use of a rotating mandrel [130], gap electrospinning [131], and magnetic electrospinning [132,133]. The description of these methods and their specific advantages are listed in Table 1.1. In the past decade, a variety of new electrospinning processes have been developed with the aim of generating more varied and complex fiber geometries. Prominent among these methods are coaxial electrospinning [134], fiber blending [135,136], emulsion electrospinning [137,138], and edge electrospinning [139,140]. The description of the new electrospinning processes and specific advantages of each are listed in Table 1.1. In coaxial electrospinning, since the fiber generation process occurs rapidly, there is no possibility of any mixing of the core and shell polymers. Examples of electrospinning using fiber blending include creation of a polyurethane (PU)-gelatin bicomponent fibrous scaffold for wound dressing applications [146] and formation of an RGD peptide cell-adhesive gradient through the depths of a scaffold to direct cellular migration [147]. Fiber blending represents a future area of advancement for electrospinning and its use in creating more in vivo–like scaffolds.

Emulsion electrospinning is mainly used for delivery applications, where drugs, enzymes, growth factors, etc., are often emulsified within hydrophobic polymers, so that their bioactivity is retained and sustained release can be achieved [137,138]. To address the issue of speed (electrospinning is a slow process), a method called edge electrospinning [139,140] has been developed. An examples is a study by Thoppey et al. who used edge electrospinning of polycaprolactone and saw an increase in the production rate by about 40 folds [140]. New electrospinning methods that have been designed to achieve complex mesh composition and architectures include coelectrospinning [141.142], hydrospinning [143], and 3D electrospinning [144,145]. One example of coelectrospinning is when a natural polymer is used along with a synthetic polymer, the cellular behavior and the mechanical properties of the resulting scaffold can be independently controlled by altering the weight ratio of each material [142]. Examples of hydrospinning include a study where

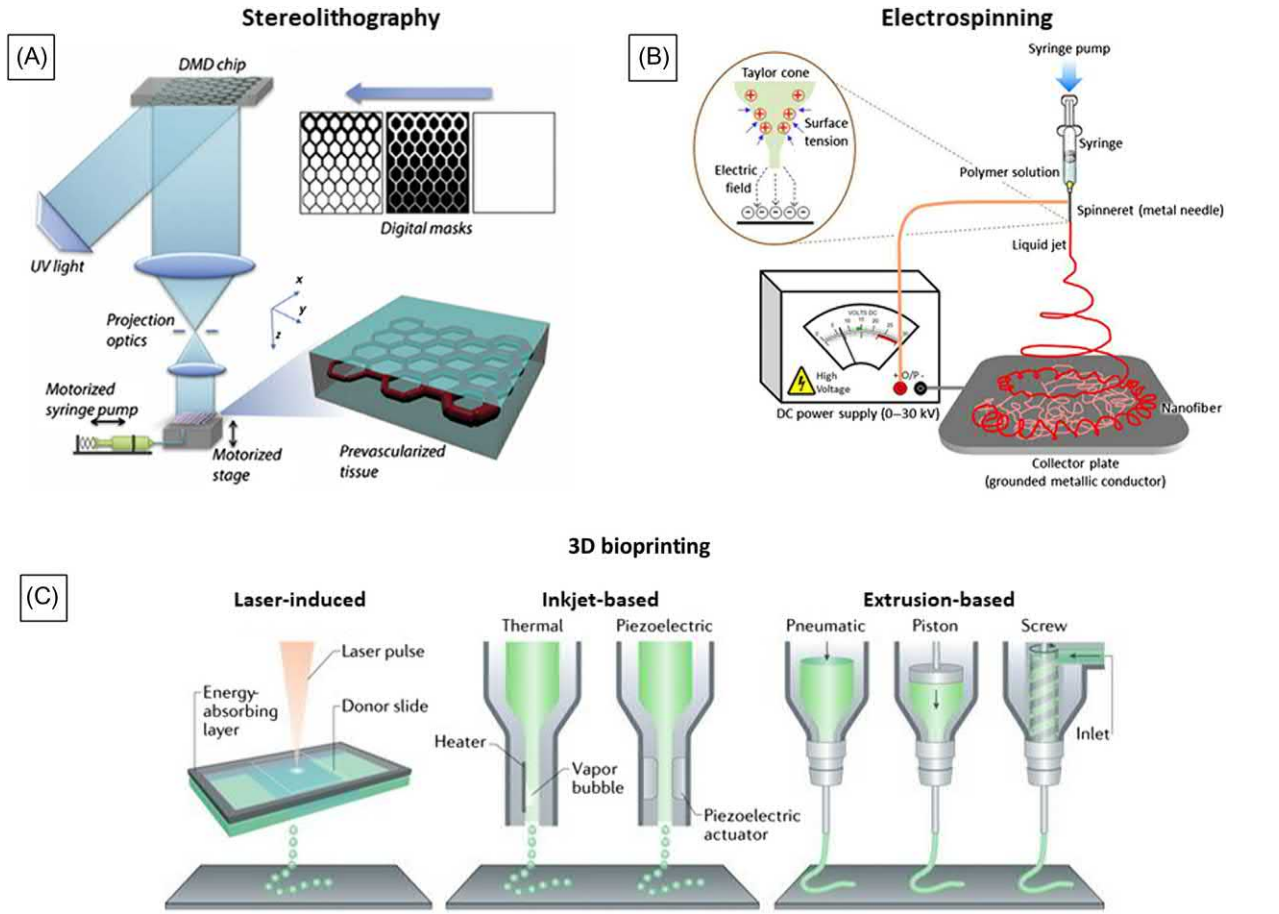

FIGURE 1.4 Different types of biofabrication technologies. (A) Stereolithography, showing an example with bioprinting of a prevascularized tissue; (B) electrospinning; (C) different types of 3D bioprinting, including laser-induced bioprinting, inkjet-based bioprinting, and extrusion-based bioprinting. *3D*, three-dimensional. *Reprinted with permission from (A) Zhu W., Qu X., Zhu J., Ma X., Patel S., Liu J., et al. Direct 3D bioprinting of prevascularized tissue constructs with complex microarchitecture. Biomaterials 2017; 124:106-115. doi: 10.1016/j.biomaterials.2017.01.042. ©2017 Elsevier Ltd.[118]; (B) Ghosal K, Chandra A, Praveen G, Snigdha S, Roy S, Agatemor C, et al. Electrospinning over solvent casting: tuning of mechanical properties of membranes. Sci Rep, 2018, 8, Article number: 5058. ©2018 Springer Nature Publication AG [119]; (C) Moroni L, Burdick JA, Highley C, Lee SJ, Morimoto Y, Takeuchi S, et al. Biofabrication strategies for 3D in vitro models and regenerative medicine. Nat Rev Mater, 2018, 3:21–37; ©2018 Springer Nature Publication AG [120].*

scaffolds with high porosity were created that allowed for better cellular infiltration [143] and another where anisotropic scaffolds with layers that altered in alignment were created for better tendon TE [148]. Examples of 3D electrospinning include fabrication of interconnected tubes with different structures and patterns [144] and reconstruction of outer ear using an ear-shaped collector to generate the scaffolds [145]. Achieving optimum cellular infiltration within electrospun scaffolds has been a challenge and an intrinsic limitation of the electrospinning method. A variety of methods that are being developed to improve cellular infiltration and direct their behavior within electrospun scaffolds include variations of the electrospinning process (as discussed above), use of postprocessing procedures, and incorporation of biochemical cues.

Another way to decrease packing density and increase pore size within electrospun scaffolds is by using sacrificial components during coelectrospinning [149,150]. After fabrication, the sacrificial component is removed by treating the scaffold with an aqueous solution. Postelectrospinning processing (postprocessing) of the scaffolds is another practical way to enhance cellular penetration. Postprocessing methods include use of laser ablation to pattern pores into the scaffold [151], use of a metal comb to separate fibers after the electrospinning [152], and used of ultrasonic energy to mechanically agitate fibers immersed in a liquid [153]. Till now, the most effective method to enhance cellular infiltration within electrospun scaffolds has been the use of dynamic cell culture. Dynamic cell cultures have been achieved for this purpose by using either simple setups such as an orbital

TABLE 1.1 New methods and recent trends in electrospinning.

Trend	Method	Description	Advantages	References
Modification of the electrospinning setup	Rotating mandrel	A rotating mandrel is used to orient and collect fibers	Controlling fiber alignment	[130]
	Gap electrospinning	Combinations of electrodes and/or electric charges are used to create varied fiber alignments	Creating multilayered scaffolds with varied fiber alignment	[131]
	Magnetic electrospinning	Polymer solution is magnetized and a magnetic field is used to stretched the fibers and align them across a gap	Controlling fiber alignment; collecting fibers over raised topographies; creating wavy or curly fiber architectures	[132,133]
New electrospinning processes	Coaxial electrospinning (also called core–shell electrospinning)	Two separate polymer solutions are fed into concentrically arranged needles, resulting in a compound polymer jet where the core and shell polymers are separate	Creating fibers from difficult to use materials; electrospinning immiscible blend of polymers; creating hollow fibers	[134]
	Fiber blending	Two or more polymer solutions fed through a mixing head to enable complete blending of the materials	Incorporating properties of two or more different polymers in a single fiber; creating bi- or multicomponent fibers; generating gradients of components across the depth of a scaffold	[135,136]
	Emulsion electrospinning	Bioactive reagents are encapsulated within hydrophobic polymers or a dissolvable material is emulsified within the primary polymer and later removed	Incorporation and controlled release of bioactivity compounds within electrospun fibers; creating porous fibers	[137,138]
	Edge electrospinning	High voltage is applied to multiple fluid streams at once so that multiple jets can be produced from a single spinneret	Substantially increases the fiber generation and collection speed; suitable for industrial production of electrospinning	[139,140]
Methods to achieve complex mesh composition and architectures	Coelectrospinning	Simultaneous electrospinning from multiple spinnerets onto the same collector	Fabricating composite scaffolds that have more than one type of polymer fiber; controlling ratio and gradient of different materials within the scaffold; increasing mesh pore to allow for cell infiltration	[141,142]
	Hydrospinning	Electrospun fibers are collected on the surface of a water bath instead of the traditional conductive metal	Creating scaffolds with layers that have altered alignments; increasing scaffold porosity for better cellular infiltration	[143]
	3D electrospinning	3D collectors are used to form fibrous architectures such as multilayered stacks or tubes		[144,145]

3D, Three-dimensional.

shaker [154] or a complex system such as a flow perfusion bioreactor [155].

Inkjet three-dimensional bioprinting

3D bioprinting is a process in which biomaterials or biomaterials combined with cells are deposited in predefined patterns, layer-by-layer using a bottom-up assembly approach to create a 3D biological structure [8]. This technology is making it possible to recapitulate the structure, composition, and complexity of human tissues and, ultimately, may lead toward whole organ engineering for clinical use. Inkjet 3D bioprinting is a method that uses droplets of inks (polymers, cells, or combinations of the two) to create 3D cellular or tissue structures (Fig. 1.4C). The first attempts to print live cells using then inkjet method was carried out using a modified commercially available inkjet printer [8]. However, due to severe limitations in that approach, special 3D printers were designed to dispense cells and biological materials into a desired pattern using droplets that were ejected via thermal or piezoelectric processes. The main advantages of inkjet printing is their high resolutions (5−50 μm), high cell viability, high print speeds, and low costs. However, there are problems too, including less control of droplet directionality, unreliable cell encapsulation due to the low viscosity of the ink material, restrictions on the viscosities of materials that can be used, and limitations of vertical printing. Some examples of tissue fabrications using inkjet include full thickness skin models with pigmentation [156], cardiac tissue with a beating cell response [157], neural tissue [158], and a bone-like tissue [159].

Extrusion three-dimensional bioprinting

Extrusion-based bioprinting is focused on the printing of biomaterials, cells, bioactive molecules, or combinations thereof by extruding continuous cylindrical filaments using pneumatic, piston-driven, or screw-assisted systems (Fig. 1.4C). This technology supports precise deposition of materials/cells and formation of complex 3D structures. Extrusion-based bioprinted structures have better structural integrity compared to inkjet-printed structures and can be used to form porous 3D scaffolds. The resolution that can be achieved with extrusion-based systems are relatively low as compared inkjet or laser-based systems, but anatomically shaped structures can best be generated using this technology. In the past several years, extrusion-based 3D bioprinting has been receiving considerable attention for creating artificial tissues or organs [160,161]. This technology has been supported by development of new bioink materials that can mimic many features of native ECMs while at the same time support cell adhesion, proliferation, and differentiation [162,163].

Extrusion-based bioprinting systems (bioprinters) rely on continuous dispensing of polymer and/or hydrogel filaments through a micro-nozzle (about 25−300 μm or larger pore diameter) and positioning them according to a 3D digital design file, via computer-controlled motion either of the printing heads or collecting stage or both. New technological advances in the past decade include development of advanced extrusion 3D bioprinters, such as the integrated tissue-organ printer (ITOP) at the Wake Forest Institute for RM [160]. The ITOP used clinical imaging data to print simple-to-complex human-scale tissue constructs using biomaterials, cell-laden hydrogels (bioinks), and a multinozzle extrusion system. Examples of tissues printed and implanted in vivo include bone, cartilage, skeletal muscle, cardiac tissue, skin, liver, kidney, bladder, lungs, and trachea. Another advancement was printing of micro-channels within tissue constructs that supported the diffusion of oxygen and nutrients to cells within the construct. Although many challenges still remain for 3D bioprinting of complex human organs, the ability to print using multiple materials and cells simultaneously and create human-sized constructs represents a significant progress in realizing the goal of TE. Another example of an advanced 3D bioprinting system is the one described by Liu et al. [164]. By combining seven capillaries in a single print head, each of which is connected to a different bioink reservoir, this bioprinter can extrude multiple bioinks in a continuous manner. In addition, each capillary can be individually actuated and controlled and fabrication of complex constructs is made possible by fast and smooth switching among different reservoirs. This 3D bioprinter addresses the limitations associated with conventional multihead printers where multimaterial printing can compromise fabrication speed, complexity, or both. Although this system requires further optimizations and validations, it is a good example of the type of disruptive technology needed to advance in the field of TE.

For creating human size tissues, extrusion-based 3D bioprinting is the most suitable technique. The printing material forms an important component of this strategy, where they primarily provide cells with the right environment to proliferate, differentiate, and form tissues. Therefore a rational bioink design approach for specific applications will be crucial to the success of the bioprinting strategy. New trends in bioinks for 3D bioprinting include self-healing and shear-thinning hydrogels that are based on supramolecular assembly of nanoparticles, small molecules, or macromolecules. These materials have unique rheology and gelation properties, which can be tailored according to need and also the printing processes [165]. Such materials have been used before as injectable cell carriers and for cell encapsulation [166], but their use to formulate bioinks is a recent development.

Examples include a study by Li et al. who have described a shear-thinning hydrogel based on a polypeptide−DNA derivative [167]. Loo et al. have developed a peptide bioink using hexapeptides that self-assemble into stable nanofibrous hydrogels [168], while Schacht et al. developed a shear-thinning bioink hydrogels using recombinant spider silk proteins [169]. To produce 3D-printed cell-laden constructs, this spider silk proteins-based bioink does not require any additional components, while at the same time, it shows a good printing fidelity and cell compatibility. A hyaluronic acid (HA) bioink that crosslinks through supramolecular assembly is described by Burdick et al. [170]. This hydrogel displays shear-thinning and self-healing properties, where the hydrogel flows due to the shearing stress applied during the extrusion process, while after printing it rapidly solidifies without any further trigger. Further stability of these hydrogels can be increased by introducing methacrylates into the HA macromers, thereby allowing for printing of complex 3D structures or perfusable channel patterns without using any scaffolding materials. Further iterations to this HA bioink include a dual-crosslinking hydrogel system, where guest−host bonding was followed by photopolymerization [171]. This new bioink formulation was also use to print stable 3D structures without using any scaffolding materials.

Use of polymeric hydrogels for 3D bioprinting has attracted substantial attention in recent years due to their tunable properties and structural similarities to native ECM. Some of the polymers used to make hydrogels include PEG, polyesters, poly(*N*-isopropyl acrylamide), and polyphosphazenes [172]. The advantage of using synthetic polymers for making bioinks is that their physicochemical properties are more controllable compared to naturally derived polymers. Examples include bisilylated PEG-based hydrogels that can crosslink through Si−O−Si bond formation without need for any crosslinking agents [173]; a photocrosslinkable acrylated PEG-fibrinogen based bioink that can form hydrogel networks through calcium-mediated ionotropic interactions and then photo-crosslinked [174]. Similarly, Lorson et al. have developed thermos-reversible supramolecular hydrogels and used them as bioinks [175]. Hybrid bioinks are a new trend in 3D bioprinting, where a biocompatible polymer is combined with a material that imparts unique properties to the bioink for a specific application. An example is a hybrid bioink made from PU and graphene oxide (GO), where the GO specifically supported the formation of neural tissues [176].

Recently, researchers have been developing decellularized ECM (dECM) as bioinks for 3D printing of tissue and organ structures. Similar to the decellularized tissues, the dECM-based bioinks can more accurately recapitulate the biochemical microenvironments of the native tissue ECM compared to just using biomaterials [177]. Studies have shown that 3D-printed tissue made using dECM-based bioink support better cell proliferation, differentiation, maturation, and overall therapeutic effects in vivo after transplantation [178]. Examples of use of dECM-based bioinks for TE include fabrication of stem-cell-laden cardiac tissue patches for the treatment of myocardial infarction models [179] and hepatic tissue for liver regeneration [180]. Research and development of dECM-based bioinks are work in progress and it would be interesting to see how further developments in this direction can help create structurally and functionally relevant tissues and organs.

Nanotechnology has been making its way into several RM applications during the past decade [181]. Some of these nanotechnologies have been used in the field of biofabrication [182,183], such as in the nanocomposite bioinks (nanoinks) with tailored properties for specific applications. Examples include a bioactive DNA/HA-coated single-wall carbon nanotube (CNT)-based nanoink for printing two-dimensional (2D) and 3D flexible electronics [184]. Using a two-step process, 3D structures with conductive patterns were printed on several supports, including within hydrogels. Another example is the study by Lind et al., who 3D printed "cardiac organs-on-chip" using a combination of thermoplastic PU filled with carbon black nanoparticles (conductive inks) with other inks [185]. The printed structures within then chip conferred various properties such as biocompatibility, high conductance, and piezoresistivity. Jakus et al. used nanoinks composed of poly(lactide-*co*-glycolide) (PLGA) and graphene for printing 3D neuronal conduits that promote neural regeneration [186]. In addition to biocompatibility, neurogenic differentiation of the seeded human mesenchymal stem cells (hMSCs) was demonstrated on these materials along with formation of axons and presynaptic-like terminals.

Spheroids and organoids

It is now well-known that 2D cell culture environment can make it difficult to control cell−cell and cell substrate interactions in natural tissue, thereby presenting limitations in recreating biological and physiological features of human tissues and organs. To address this, researchers have developed different types of 3D scaffolds using natural and synthetic polymers. Hydrogels have been one of the most commonly used 3D scaffolds for cell culture and TE due to their high water content, ECM like microstructure and biocompatibility [187,188]. However, use of simple hydrogels could not fully recreate the complex microenvironment of many higher order tissues and organs. One of the basic question being asked today in TE is how precisely the physical, chemical, and biological

properties (if applicable) of a 3D scaffold can support or regulate cell function.

The regulation of the hydrogel environment is a promising method for controlling cellular behavior in three dimensions. One example of such an approach is a study by Caballero et al., who used a pattern with microgrooves to engineer anisotropic fibroblast-derived matrices [189]. Fibroblasts seeded into this matrix showed in vivo−like phenotype such as alignment, spreading, and migration. In another study, Trappmann et al. devised a way to control the swelling of a dextran hydrogel by attaching a methacrylate (a hydrophobic pendant group) to dextran (the hydrophilic polymer chain in then hydrogel) and found that the swelling of these hydrogels could be reduced from 55% to 0% (no swelling) [190]. In addition, insertion of a di-cysteine peptide sequence allowed for a partial degradation of the hydrogel through cleavage by MMP that also supported endothelial cell migration and angiogenesis. Success of such approaches provides support to more innovations in 3D cell culture and tissue biofabrication, all of which will be advancing then field of TE in the coming years.

Among 3D culture systems, cellular spheroids have been attracting more attention lately. These are 3D complexes composed only of cells, where the spheroid is formed based on the self-assembling tendencies of these cells [191]. Cell spheroids offer several advantages over 2D cell cultures, including better cell−cell interactions and diffusive mass transport. Therefore they can not only be used for investigations of physiological and developmental processes but also as building blocks for TE (Fig. 1.5). For TE, spheroids of multiple cellular origins can be combined or multiple cell types can be incorporated in a single spheroid using coculture. They are produced using a variety of devices such as microwell or hanging drop plates. However, spheroids made this way have features that are somewhat different from the structure of the native tissues. To address this, new ways of spheroid generation are being explored. One example is using phosphoproteins and glycoproteins to generate supramolecular nanofibrils and then induce self-assembly of fibroblasts [193]. Spheroids produced this way had a more tissue-like form. In another study, fragmented nanofibers as a physical artificial support were injected into the spheroids to control cell function [194]. In addition, using this approach, the researchers could create larger spheroids (~800-mm diameter) compared to the spheroids that were made only of cells. Another example of spheroids application is for studying the physical stimuli that may occur within tissues. Dolega et al. measured mechanical stress within spheroids by injecting an ultrafine polyacrylamide microbeads as a pressure sensor [195]; while Cho et al. made multicellular spheroids composed of human brain vascular pericytes, primary human astrocytes, and human brain microvascular endothelial cells in agarose gel and used it for blood−brain barrier studies [196].

Applications of cellular spheroids include cancer research, disease modeling, and in vitro platforms for drug/toxicity testing. Although spheroids have been around for more than 10 years, their application for TE has not been common. One reason is that they are primarily used for mimicking microniches, plus the control of spheroids during culturing has been an issue. Also, if a spheroid is larger than a certain size, necrosis occurs in the core, thereby reducing their usefulness for mimicking structurally complex and multicellular tissues. However, the regenerative potential and fusion capacity of these spheroids can be improved by in vitro preconditioning or by incorporation of biomaterial components. Fusing them

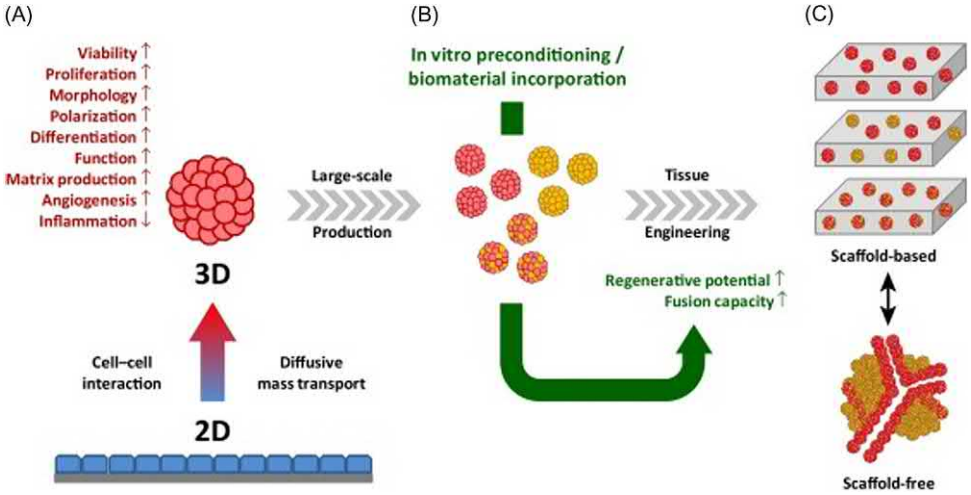

FIGURE 1.5 Use of cellular spheroids, from studying basic biological processes to tissue engineering. (A) Some of the physiological and developmental processes that can be investigated; (B) improving the regenerative potential and fusion capacity of spheroids; (c) the two types of tissue engineering strategies using spheroids. *Reprinted with permission from Laschke MW, Menger MD. Life is 3D: boosting spheroid function for tissue engineering. Trends Biotechnol 2017;35(2):133−44. doi: 10.1016/j.tibtech.2016.08.004. © 2017 Elsevier Ltd. [192].*

together can generate scaffold-free macro-tissues, while seeding them on scaffolds can generate in vivo–like engineered tissues.

A more complex spheroid type are 3D tissue organoids. An organoid is an extended cellular spheroid that has a physicochemical environment very similar to the tissue it is representing. The generation of organoids can be considered as one of the major technological breakthroughs of the past decade. Organoids can be generated using different cell sources (such as autologous cells, ES cells, iPSCs) and self-organization or fabrication methods (hanging drop plates, ultralow attachment plates, agarose-coated plates, or ECM surface culture) (Fig. 1.6A and B). Organoids can display various biological features seen in vivo, such as tissue organization, regeneration, responses to drugs, or damage. Examples of some of the tissue-specific organoids that have been developed include liver [200,201], lung [202], pancreas [203], prostrate [204], intestine [205], heart [206], brain [199]. The development organoids representing the nervous system had been a challenge so far. However, recently, Birey et al. successfully fabricated human subpallium and human cortical spheroids [207]. They showed that γ-aminobutyric-acid-releasing (GABAergic) neurons could migrate from the ventral to the dorsal forebrain and integrate into cortical circuits. Success of such efforts provide confidence that it is possible to create micro-tissues that can more closely mimic structure and physiological aspects of complex tissues.

3D bioprinting has become a popular way of creating engineered tissues to be used both for studying the basic tissue biology or pathology and for repair or regeneration in vivo. A recent application of 3D bioprinted tissue is for toxicity testing, drug screening, and development with the aim of reducing or eliminating the use of animals for these purposes. Many types of tissues are being 3D bioprinted for in vitro use. One interesting example is a multicellular 3D hepatic tissue by Chen et al., who used methacrylated gelatin (GelMA) and glycidyl methacrylated HA, with human iPSCs, adipose-derived stem cells (ADSCs), and human umbilical vein endothelial cells (HUVECs) as the bioink to print microscale hexagonal architectures that mimicked the native hepatic microenvironment [208]. In addition to better morphological

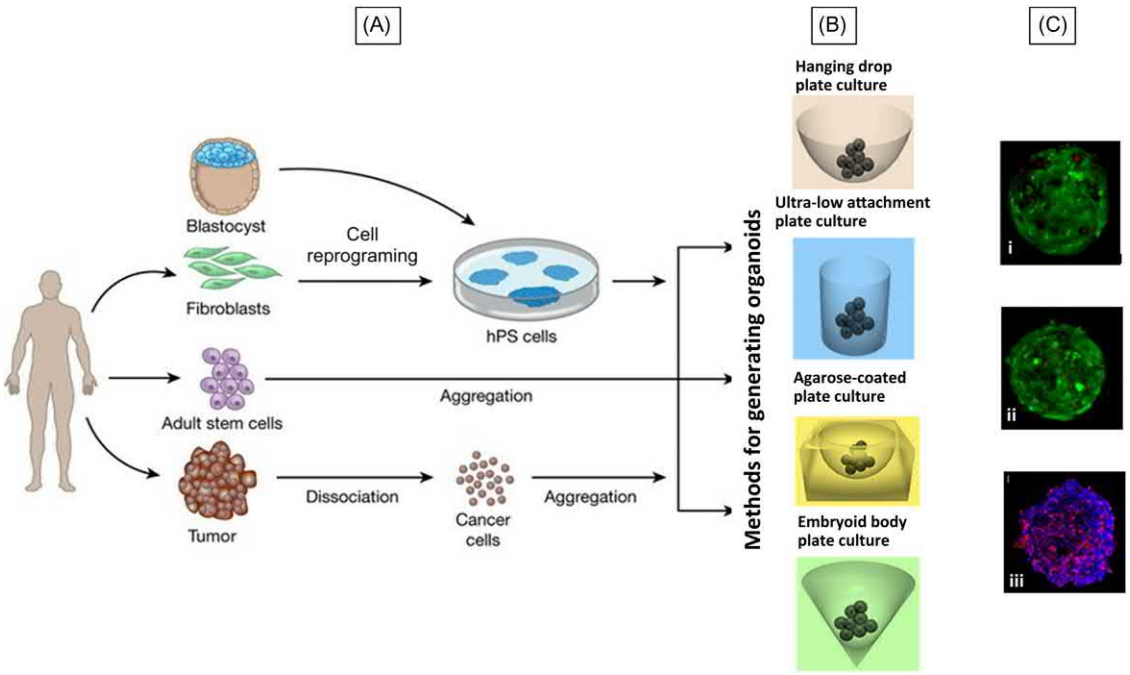

FIGURE 1.6 Different cell sources and methods used to generate organoids. (A) Embryonic stem cells, keratinocyte-derived iPSCs, adult stem cells, or even cells from tumors can be used to make tissue or disease-specific organoids; (B) generating organoids using different platforms; (C) photomicrograph of liver organoid stained with Calcein AM and Ethidium heterodimer (i), cardiac organoid stained with Calcein AM and ethidium heterodimer (ii), and six cell types containing human brain cortex organoid stained with CD31 (iii). *iPSCs*, Induced pluripotent stem cells. *Reprinted with permission from (A) Pasca SP. The rise of three-dimensional human brain culture. Nature 2018;553:437–45. doi: 10.1038/nature25032. ©2018 Springer Nature AG [197]; (C) (i and ii) Forsythe SD, Devarasetty M, Shupe T, Bishop C, Atala A, Soker S, et al. Environmental toxin screening using human-derived 3D bioengineered liver and cardiac organoids. Front Public Health 2018; 6:103. doi: 10.3389/fpubh.2018.00103 [198] and (iii) Nzou G, Wicks RT, Wicks EE, Seale SA, Sane CH, Chen A, et al. Human cortex spheroid with a functional blood brain barrier for high-throughput neurotoxicity screening and disease modeling. Sci Rep 2018;8, Article number: 7413. for ©2018 Springer Nature AG [199].*

organization, this 3D-printed liver also showed increased metabolic secretion, enhanced cytochrome-P450 induction, and higher liver-specific gene expression.

Imaging technologies

Multiscale digital imaging data is becoming a critical part of many TE strategies that are aiming to recreate complex tissues and organs. These digital data act as blueprints upon which design and fabrication strategies of neotissues and neo-organs are based. A great deal of anatomical data already exists, for example, in the many medical computed tomography (CT) and MRI images, which can be harnessed for generating 3D computer-aided design (CAD) models of implants and tissues for biofabrication. Recent advances in bioimaging are also allowing for collection of data to create high-resolution designs for TE. For example, CLARITY is a technique by which the lipid content of tissues are removed, leaving behind a transparent, porous hydrogels−like tissue which maintains the original intact structures [209,210]. This technique can be used to visualize a tissue's structure and molecular composition at very high resolutions (as high as 70 nm) [211]. In addition, the ever increasing repertoire of MRI, contrast-enhanced nanotomography (nano-CT), histological data (including immunohistochemistry), and molecular imaging are providing large-scale structural information as well as fine molecular-level information (such as distribution and interaction of cells through a tissue or organ). Such information can be used to design multiphasic structures that have both structural and biochemical heterogeneity and in vivo−like organization.

Tissue neovascularization

Vasculogenesis and angiogenesis are two basic and critical processes by which vasculature forms during development. For tissue and organ bioengineering, attempts are now being made to recreate these processes to generate vascular networks [212]. In bioengineered tissues, the diffusion limit of oxygen is typically 100−200 μm and thicker tissue without an intact vasculature for oxygen and nutrient transport will face necrosis. In addition to creating structural vessels, vascular engineering designs will also have to ensure normal cellular metabolism within these structures, be permeable to allow extravasation of cells, maintain an open-vessel lumen during remodeling, and also promote integration with the host vasculature [213]. Efforts are underway in this direction, which include a study by Lewis et al., who 3D printed a cell-laden, thick (>1 cm), vascularized connective tissues that could be perfused and sustained in culture for >6 weeks [214]. The 3D vascular networks were created using a pluronic fugitive ink and the vessels were lined with HUVECs and MSCs. To demonstrate the functionality of then vasculature, osteogenic differentiation of hMSCs within the construct was induced by osteogenic media delivered through the vasculature. In addition to highlighting the importance of a functional vasculature for engineering thick tissues, this study also provides a design platform to build other tissues with functional vasculature.

Jia et al. demonstrated an alternative method to 3D print vascular networks, one that does not use sacrificial inks [215]. Here, a newly designed multilayered coaxial nozzle device was used to 3D bioprint perfusable vascular channels using a specially designed cell-responsive bioink. Printing was carried out using a trilayered coaxial nozzle, while the bioink contained sodium alginate, gelatin methacryloyl, and four-arm poly(ethylene glycol)-tetra-acrylate. A multistep crosslinking process, followed by removal of the alginate using Ca^{2+} resulted in creation of a layered, perfusable hollow tubes. Although long-term perfusability of this construct was an issue, this approach represents an advancement over the conventional strategies to engineer vasculature in tissue constructs. Anastomosis of prevascularized 3D tissues with host vasculature are also being investigated. Examples include studies by Zhu et al., who 3D printed tissues where GelMA polymer was used along with mesenchymal cells and HUVECs to form hydrogel networks representing vasculature by photo-crosslinking [118]. On in vivo implantation, anastomosis between the host circulation and the prevascular tissues construct was observed where blood vessel growth were seen into the construct having red blood cells. For bone TE, prevascularized bone scaffolds were generated using bioactive bioinks, as described by Kuss et al. [216]. To from then vascular networks, GelMA and acrylated HA with HUVECs and human adipose-derived MSCs were used. In vitro studies showed that the prevascularized construct generated more capillary-like structures when compared to nonprevascularized tissue, and when implantation in vivo in mice, anastomosis with host vasculature was observed along with microvessel and lumen formation.

Bioreactors

Bioreactors are critical for engineering new tissues. They are used to recapitulate physiological environments in which cells exist in vivo: expand cells for potential clinical use, to test potential new treatments in an in vivo−like environments, and to establish new therapeutic targets. Significant improvements in design and construction of bioreactors have been made over the past decade. Some of these systems can support growth of whole tissues of 3D bioengineered constructs, coculture of cells of different phenotypes, generate dynamic culture

conditions which facilitates mass transfer, and have advanced sensors and imaging capabilities allowing for real-time monitoring of physiological parameters. Whilst more sophisticated bioreactor can more accurately recreate the complex cell/tissue microenvironments, they are challenging to create and operate. In contrast, simpler bioreactor designs can recreate only selected conditions but are more operationally robust. Therefore a compromise between complexities versus essential functionality needs to be determined based on the application and the desired end-product. Perfusion bioreactors are being used to closely simulate the in vivo environments for TE. In a perfusion bioreactor, the media flow is either cross, downwards, or in a specific pattern that generated some kind of microgravity environment. Fluidized bed bioreactors and ones with rotating wall can achieves greater mass transfers, which are beneficial to the tissue or organ being conditioned. In one study, using a rotatory cell culture system, Crabbe et al. [217] improve reseeding of decellularized lung tissue. However, if the flow in the bioreactor is not optimized, it can have negative effects. Other factors that can have important impact on tissues being cultures in bioreactors include the scaffold's architecture, oxygenation, mechanical stimuli, and sheer stress. The design of the scaffold used for making the tissue construct will impact mass transfer during culture. The scaffold pore size not only affects the cell seeding but also distribution of nutrients and gas exchange during culture. Therefore the original scaffold design should take into consideration the thickness, pore size, and interconnectivity. In general, decellularized tissues are supposed to offer an ideal scaffold environment for TE, because they are derived from the same tissue they will be replacing.

As for assisting cell seeding within scaffolds, some physical methods such as electromagnetism or acoustics have also been used [218,219]. In bioreactors where the culture media is the only nutrient source, the presence of a suitable oxygen tension is critical. Hence, balancing the oxygen environment should be an integral part of bioreactor design. Use of oxygen delivery agents such as perfluorocarbons [220] or artificial red blood cells can help create a balanced oxygen environment, while use of fluorescent oxygen sensors can help control this environment. Use of mechanical stimuli to condition cells and tissues in artificial environments has been done since a long time. The types of mechanical stimuli can include shear stress, compression, stretch, or pressure loads. With advances in bioreactor engineering, it is now possible to engineer such capabilities into the bioreactors. At the cellular level, these stimuli activate mechano-transduction cellular signaling pathways and causes changes such as focal adhesions, cell-to-cell contacts, and changes in gene expression. Cultured tissues such as blood vessels, muscles, and ligaments would need such stimuli to develop into in vivo–like tissues before implantation. For example, in cardiac tissues, mechanical stretch and/or electrical stimuli lead to synchronized contractions [221], while application of cyclic stretch in muscles enhances ECM protein content, among other things [222]. Shear stress in a dynamic environment may not represent the physiological state for some tissues and may impact their performance. An example is liver, where the hepatocytes are shielded from shear even though the blood flow through the portal vein at a rate of 1200 mL/min.

Organ-on-a-chip and body-on-a-chip

The continuous rise in drug development cost and increase in the number of drug candidates failing in clinical trials, despite promising results from traditional preclinical model systems, have prompted development of better physiologically relevant and predictive in vitro/ex vivo systems. Therefore better and physiologically relevant in vitro models are needed that better predict drug efficacy and safety in humans and which can be used in a high-throughput way. More recently, the engineering of complex tissues in vitro has been supported by two integrated approaches: (a) organoids and (b) microfluidics-based organ systems known as "organs-on-a-chip" or "body-on-a-chip" [223]. 3D organoids have been developed to recapture the phenomenon of controlled cell growth to appropriate length scales in three dimensions, which happens in body tissues. Use of organoids is now expanding beyond basic research to disease modeling, drug discovery/ development, and personalized medicine. Also, organoids can be easily integrated with chip models via microfluidics. The organoid-based "organs-on-a-chip" systems can surpass traditional "cells-in-a-dish" models with regards to recapitulating in vivo structural and physiological microenvironments [224]. Furthermore, different tissue-specific organoids can be connected in an integrated circuit format using microfluidics to create a "body-on-a-chip."

New technologies that are permitting development of complex biological systems with control over the physical and chemical microenvironment include microfluidics. Microfluidics can allow us to create 3D microphysiological systems, such as organs-on-a-chip or body-on-a-chip. These systems can allow us to study living tissues and organs in a more complex way. Microfluidics-based 3D multifunctional systems can also support cell and organoid cultures, where control of parameters such as nutrient low, oxygen gradients, and drug/biologics distribution can be used for accurate high-throughput testing and environment sampling applications. Microfluidic-based systems have contributed significant to the in vitro modeling of tissues such as heart [225], kidney [226], and blood–brain barrier [227]. Here, well-defined cellular

environments closely mimic a physiological niche for better understanding of the biological mechanisms operating within that particular tissue.

Recreating the highly complex structures of organs is a tremendous engineering challenge. A better way for studying the physiology, drug response, or chemical toxicity in a single organ or an organ system is to create miniaturized version of these organs that capture their functions. Organ-on-chip systems represent an effort in that direction. Using these systems, cell function can be quantitatively studied in response to precisely controlled physicochemical cues. A higher version of this is body-on-chip, where multiple tissues representing different organs are placed on a single-chip format and interconnected by microfluidic channels representing vasculature. In case of drugs, it is well-known that its metabolic products can sometimes have adverse effects on body systems other than the target [228]. By only studying an isolated organs in vitro, the vital information that can emerges from synergistic effects on the entire organ systems can be lost. Use of body-on-chip can help capture that information, not only with reference to drug interactions, but also for disease etiologies or basic biological processes [229,230]. The use of 3D bioprinting technologies are helping to print complex tissues with diverse types of matrix, cells, growth factors, etc. The tissues in the organ-on-chip and body-on-chip can now be 3D printed at high resolutions and in a highly replicative manner. This has supported advancement of the organ-on-chip and body-on-chip systems from investigative stage to use as a reliable diagnostic tool.

Integration of nanotechnology

Nanotechnology is the understanding and control of matter at the nanoscale, at dimensions between approximately 1 and 100 nm, where unique phenomena enable novel applications (https://www.nano.gov/nanotech-101). Based on the application, nanotechnology can involve either measuring, modeling, imaging, or manipulating matter at then nanoscale. In the field of TE, use of nanoparticles has been shown to significantly enhance the physical and biological properties of scaffolds and also serve various functions based on the application [231]. Examples of nanoparticles used in TE include titanium oxide (TiO_2) nanoparticles, silver nanoparticles (AgNPs), gold nanoparticles (GNPs), CNTs, magnetic nanoparticles (MNPs), and GO nanoparticles. Titanium oxide nanoparticles have been primarily been used to improve the mechanical properties of TE scaffolds but have also been observed to enhance cell proliferation. Nanocomposite polymers with TiO_2 have shown superior mechanical properties compared to plain polymers for TE applications [232,233], including a biodegradable patch that was used to reinforce scars after myocardial infarction [234]. When 3D-printed scaffolds made from PLGA were used to culture human ES cell−derived cardiomyocytes, incorporation of TiO_2 nanoparticles within the scaffolds resulted in enhanced proliferation of these cells. GNPs are widely used in TE. Electrical properties of scaffolds have been enhanced using GNPs, particularly for cardiac TE [235]. Examples of such studies include better striation and enhancements in electrical coupling of cardiac cells when decellularized matrices incorporating GNPs were used [236]. In other studies, use of electrospun scaffolds containing GNP improved the treatment outcomes of infarcted heart [237].

For bone TE, GNPs have been shown to enhance osteoclast formation from hematopoietic cells [238], promote osteogenic differentiation in cells such as MSCs, and MC3T3-E1 (osteoblast precursor cells) [239]. Since, bone morphogenetic proteins (BMPs) is the primary biological factor that can modulate these biological response, GNPs can be viewed as replacements for BMPs in certain situation and can also be good candidates for inclusion in bone TE applications. Another example where GNPs have substituted the use of growth factors is for direct stem-cell differentiation. In a study, scaffolds incorporating gold nanowires and GNPs could direct stem-cell differentiation, without using growth factors [235]. This is another example that shows that certain nanoparticles can be used to substitute use of biological factors, which are known to have various side effects when applied in the body. Nanoparticles could be a new tool for controlling TE outcomes. Infection of a surgical site is a wide spread problem in clinical medicine even after availability of many ways to prevent and treat it. For tissue-engineered constructs, most of which would be surgically implanted at the target site, one way to prevent or treat the possibility of postsurgical infection is to incorporate an antimicrobial agent within the construct itself, mostly within the scaffold. Metal oxides such as iron-oxide nanoparticles, AgNPs, and selenium nanoparticles are widely being used for this purpose. Silver nanoparticles are more popular as antimicrobial agent and have been used in TE of skin [240], bone [241], and vascular grafts [242]. In addition to antimicrobial effects, AgNPs have shown to have wound healing capabilities [243].

Recently, the application of MNPs has been explored as a versatile tool for TE. For this, the MNPs are either direct incorporation into a scaffold matrix or the cells being seeded on the scaffolds are prelabeled with MNPs by internalization [244]. Using an external magnetic field, these MNPs can now be tracked or used for guiding cells, thereby making the resulting tissue magnetically responsive. This is an economical and attractive way to monitor and stimulate cells in vitro. In one study, Gomes et al. have used magnetic scaffolds where magnetic stimulation was used to differentiate human ADSCs for tendon

regeneration [244]. On implantation into an ectopic rat model, these scaffolds also showed good biocompatibility and integration within the surrounding tissues. Cezar et al. created biphasic iron-containing scaffolds (ferrogels) for use in muscle TE [245]. On implantation into a mouse muscle injury model and remote magnetic actuation, uniform cyclic compressions were observed in the engineered muscle. Consequently, there was also a reduction in inflammation, reduction in fibrosis, better regeneration, and around three-fold increase in the maximum contractile force of the target muscle. This was a significant outcome because use of a biphasic ferrogel to stimulate and regenerate injured muscle can mean avoiding use of growth factors or cells for muscle regeneration.

In another study, Antman-Passig and Shefi [246] used collagen hydrogels with MNPs for neuronal TE. These collagens in the injectable hydrogels could be aligned in vivo by the application of an external magnetic field and the alignment could be actively controlled postimplantation. This magnetically controlled patterning supported elongation and directional growth of primary neurons, which also exhibited normal electrical activity. Another interesting application of MNPs can be seen in magnetic field–guided cell targeting. Swaminathan et al. used superparamagnetic nanoparticles (SPION) to label smooth muscle cells (derived from bone marrow mesenchymal stem cell) and deliver them to abdominal aortic aneurysms (AAA) wall [247]. The use of SPIONs also did not have any adverse effects on cells and then authors believe that this could be a new approach for in situ AAA repair.

Integration of sensors in biological and tissue-engineered systems for real-time monitoring and data acquisition is a latest trend. Millimeter-sized living tissues fabricated in a microphysiological devices format are known as organs-on-a-chip. Integrating sensors in organs-on-a-chip platforms can allow real-time tracking and monitoring of selected physiological response without interfering with the tissue/ organ function. Examples of such an approach include development of a cardiac tissue patch that integrated an electronic network within the engineered tissue [248]. The network collects information from the tissue microenvironment and can be used for electrical stimulation, synchronizing cell contraction, or drug release on demand. Similarly, Lieber et al. developed a flexible scaffold mimicking 3D nanoelectronic array for engineering a cardiac tissue for a transient arrhythmia disease model [249]. This unique scaffold was used for mapping the action potential propagation in then developing cardiac tissues as well as using controlled electric stimulation to manipulate the tissue electrophysiology. Use of such nanoelectronics containing scaffolds can not only allow for spatiotemporal monitoring and control of developing cardiac tissues, but the data collected can reveal important parameters for better engineering of cardiac tissues. In a different application, Bavli et al. integrated sensors into liver-on-chip device for monitoring mitochondrial dysfunction in native liver microenvironments [250]. In chemically caused toxicity, mitochondrial damage and dysfunction happens that can often go undetected. This sensor containing liver-on-chip device was able to monitor real-time mitochondrial respiration and stress.

Current challenges

TE as a new field was introduced more than 20 years ago; however, the current technology for engineering complex tissues for clinical use is still far from ideal. Conventional scaffold fabrication methods such as soft lithography, electrospinning, and freeze–drying usually generate reduced reproducibility between scaffolds [251] and cell deposition accuracy during the scaffold fabrication is relatively low. Development of better biofabrication technologies such as SLA, 3D bioprinting, and laser bioprinting have resulted in better scaffolds and resulting tissues, but they are still insufficient to recreate the complex structural, cellular, and functional organization of higher order tissues and organs. This will be critical not only for successful clinical translation of TE products but also acceptance of TE as a mainstream field of medicine. Many challenges to the field of TE are specific to a particular technology or process. For example, when using electrospun grafts in clinical applications, the precise and reproducible control over fiber formation, their morphology, and composition is a big challenge. The manufacturing scale-up of the electrospinning process is also a concern because of the relatively slow rate of collection of electrospun fibers. In addition, homogenous cell distributions within such scaffolds are difficult to achieve. New methods that have been developed to address this issue include multilayered electrospinning, use of low fiber packing density, cell electrospraying, and dynamic cell culture in bioreactors.

3D bioprinting is an advanced biofabrication technology that is aiding in generation of complex scaffolds and tissues for RM. Compared to conventional manufacturing methods, 3D bioprinting can be used to generate predesigned scaffolds and cell arrangement that can mimic higher order tissues within the body. Therefore this technology is highly desirable for creating products for tissue/ organ repair and regeneration. In addition, bioprinted tissues would be highly reproducible, which is a requirement for commercial production. The development of suitable bioinks is currently the bottleneck of bioprinting technology. Since the ECM as well as cellular compositions of different tissue are highly diverse, the development of a universal bioink for ubiquitous application

proves highly difficult. For successful TE using 3D bioprinting, the bioinks have to be nontoxic, immune-compatible, biodegradable, and the degradation products should be harmless to the host and should ideally support the cells in their desired organization and function. Therefore development of novel, versatile, and tunable bioinks that meet many of the above criteria would be pivotal for the success of bioprinting as a robust tissue fabrication technology. Standardization of 3D bioprinters, the bioprinting process, bioinks, product and process scale-up, testing and quality control etc. are all challenges that will have to be addresses sooner than later.

Finding a suitable cell sources and obtaining sufficient quantities of cells has always been a challenge. In addition, for clinical applications, autologous cell source are preferable, which are difficult to get in many cases. Engineering of complex tissues will also require multiple cell types in sufficient qualities, unless stem cells are being used to derive these cell types. Creating suitable bioactive scaffolds where all these different cell types can grow, interact, build their own ECM, and perform in vivo–like function is another major challenge in TE. Reducing the immunogenicity of TE scaffold is one of the most critical requirements of the decellularization strategy. This aspect might have been critical in preventing the widespread use of decellularized ECM as scaffolds in clinical applications. This is particularly true for xenogenic scaffolds. If their immunogenicity is not sufficiently reduced, they can be rejected in vivo, leading to functional failure of the engineered scaffold of tissue. Use of detergents such as SDS and Triton X-100 can significantly reduce the presence of protein-based immunogenic components [252], while the use of endonucleases such as DNAse and RNAse has been shown to remove the nucleic acid components significantly. Use of mechanical decellularization approaches such as freeze–thaw and high hydrostatic pressure (HHP) can leave behind DNA remnants, which are immunogenic. Use of more extensive wash procedures and a combination of different enzymatic methods can completely clear these scaffolds of immunogenic components.

Engineering vascular tissues continue to remain a major challenge in TE. Without proper vascularization, the lack of nutrition or buildup of waste will inevitably lead to necrosis or cell death in the engineered tissues and organs [253]. This will invariably restrict their size and complexity, and their regeneration applications. To address this, many attempts are being made to engineering vascular structures. Current strategies include generating a hollow tubular network using sacrificial filaments followed by perfusion of vascular cells into the network [254], use of a modified thermal inkjet printer to printing microvascular networks [255], use of 3D printing to generate a sacrificial filament and then embed the filament network into hydrogel with encapsulated cells [254], or use 3D bioprint vascularized structure using multiple cell types and ECM substrates [214]. Even when a tissue-engineered construct can be made with vascularization support, its integration within the host (anastomosis) is a big challenge. Immune complications can also arise during this process, resulting in rejection of the implant. Although several studies have shown that vascularized small constructs, when transplanted into immune-compromised host mice can successfully integrate with the host blood supply, the implants used were usually small (maximum) 6-mm diameter and 1-mm thick [256]. As the dimensions of the tissue exceeds beyond this number, its vascularization and integration with the host vasculature is increasingly difficult. This is indeed a bottleneck for TE and a challenge that is being addressed.

When a technology is considered suitable for clinical use, there still can be major regulatory hurdles that need to be overcome before this translation is realized. These hurdles can be associated with commercialization, clinical trials, or both. Most of the regulations are designed to ensure safety of the patients who will be recipients of these products. Regulatory assessment considers the risk profile of the technology, the materials and processes used in manufacturing, storage, transport to the bedside, etc. Furthermore, the process scale-up and approvals for clinical applications are some of the aspects that can be complex and time consuming. Another important, but often overlooked, challenge for TE is the social acceptance of this technology. Finally, the willingness of patients to accept this technology and its products will determine its success (or failure). Hence, robust ethical discussions will also be required for realistic consideration and promotion of TE for successful clinical applications.

For TE, each individual tissue or organ poses its own engineering challenge. This is due to the fact that among other things, spatial constraints, physical forces, biochemical cues, individual cell types, and their growth programs can vary widely between each tissue type. Organs are even more complex in terms of these parameters. For this reason, most of the current tissue/organ models still cannot fully replicate in vivo biology. Being mostly a single tissue/organ model, they largely miss the dynamic effects of an organism's physiological status. As for the cell sources, ES cells, adult stem cells, and iPSCs require distinct environments to effectively induce their differentiation and tissue formation. Therefore designs of engineered tissues/organs will not only require expertise in bioengineering but also progressing biological knowledge that would drive such biodesigns. Another challenge in the field of TE is the lack of knowledge about the degree of self-assembly and remodeling that the engineered tissue will undergo after fabrication or in vivo implantation. This is an area that will need further research and knowledge generation, not only for the

engineering aspect, but also from the clinical and regulatory point of view.

While use of nanoparticles in TE has shown many benefits, such as enhancement of mechanical, biological, and electrical properties, and aid in gene delivery, antimicrobial effects, and molecular imaging, many challenges still lie ahead for using them in widespread clinical applications. Concerns that need to be addressed related to use of nanoparticles include nanoparticle toxicity, carcinogenicity, teratogenicity, and their bioaccumulation inside the body over a long period of time. A compelling need exists for developing better tools and methods to assess nanoparticle toxicity, carcinogenicity, and teratogenicity because most nanoparticles used in the human body have the potential to accumulate over a long period of time. This will allow them to reach a concentration that can cause toxicity to cells, resulting in cancers or harmful effects on physiological systems such as brain and reproductive systems and possibly also on the fetuses before their birth.

During the past 10 years, the basic and applied science base of TE has made great progressed in many areas. However, the clinical and commercial successes that were initially predicted have been slow to come. The slow clinical and commercial progress of TE can be attributed more to issues other than the science and technology. Scale-up issues in biomanufacturing, few than expected clinical trials, regulatory processes and health economics are some of the reasons for this. However, many of these hurdles are being overcome and the progress is encouraging. The coming decade is expected to more advancements, including incorporation of more new technologies, evolution of smart biomaterials, widespread use of stem cells, fabrication of more complex tissues and whole organs, use of bioengineered tissue as replacement for animal testing, personalized medicine, and clinical translation of way more tissue-engineered products. Development of complex tissues and organs involves the intricate process of coordinated cell growth, differentiation, morphogenesis, and maturation of diverse populations of cells. Although the ability to engineer complex tissues and whole organs is attractive and has the potential to impact translational medicine, it is still a major challenge. To meet this challenge, knowledge from multiple disciplines such as development, anatomy, physiology, and cell biology will have to be combined with enabling technologies such as biomaterials, biofabrication (including 3D bioprinting), advanced bioreactors, 3D modeling, high-content imaging, and nanotechnology.

Future directions

Through a greater understanding of biology, technological advancement, multidisciplinary approach, and clinical studies, the fields of TE and RM have been steadily advancing. Since TE is both a multi- and interdisciplinary science, joint efforts of different specialties will be needed for the commercial and clinical success of TE products. At least for commercial applications, TE and RM should be viewed from a systems engineering perspective. Successful translation of more TE technologies will be needed for long-term success of this field and also to establish confidence in TE and RM as viable solutions to many unmet medical needs. The clinical feasibility of TE and RM products has already been demonstrated for several applications. Now the challenge is for showing their efficacy in real-world patients. To benefit a larger patient population, manufacturing of these products will have to be scaled-up, produced reproducibly with high-quality control, and made broadly available in an economical way. From a business perspective, the challenges for TE and RM will be more than just the technology. A viable business model will have to be developed that takes into consideration the technology, regulatory aspects, cost considerations, and the healthcare landscape. From the science and technology perspective, some of the most promising aspects that will drive the field of TE in the future are discussed next:

Smart biomaterials

A better understanding of the interactions of biomaterials with cells and tissue in vivo will help develop the next-generation of biocompatible materials for TE and RM applications. For example, using computational models of material surface chemistries and studying their interactions with cells can be very helpful. Clinical translation of life science-based technologies is always challenging. Same is the case with TE technologies, particularly smart biomaterials. However, considering some critical factors during the research and development phase can help ensure a better translation to clinical studies and finally to the patients. These include biocompatibility of these materials over longer time (months or years), material performance consistency, and standardization of manufacturing parameters (process scalability, batch to batch reproducibility etc.). The use of computational and machine learning technologies can also have positive benefits for design of biomaterials and improve predictions during manufacturing and their performance in vivo.

It is now known that many ECM components have natural immunomodulatory domains that bind to receptors found on immune cells, enabling their adhesion and regulating their function [257]. This is facilitated by bioactive amino acid sequences that encode specific cellular cues. Therefore it is possible to use ECM proteins and/or ECM-modeled peptides in biomaterial scaffolds to mimic the natural regulatory role of the ECM on the host immune

system. This strategy to modulate favorable immune response to can ensure functionality and longevity of TE implants. Proteins-based smart biomaterials (including composite hydrogels) can have unique applications in TE and RM and need further exploration. It is also possible to make composite hydrogels with proteins and synthetic polymers. Burdick et al. have developed a self-assembly mechanism to make such hydrogels [258,259]. These hydrogels can also be further strengthened by reinforcing with photo-reactive groups (such as methacrylate groups), so that the hydrogel crosslinking can be carried out by UV radiation [259].

Biomaterials that can respond to electrical and stimulus are also interesting for many applications. It is well known that electrical stimulation positively affects cardiac cells in culture [260] and other electrically sensitive cells such as neurons have displayed improved growth and function when cultured on conductive scaffolds [261]. Electrically conductive polymers have also been combined with proteins [262] and represent an interesting class of biomaterials that need further exploration. Since some proteins can undergo structural changes in response to mechanical environmental triggers [263], combining such proteins with other biomaterials can result in mechanically responsive smart biomaterials. Growing electrically or mechanically responsive tissues for in vitro use or for in vivo for tissue regeneration would represent another advancement in the field of TE.

Cell sources

Stem cells may have their own drawback and pose certain common challenges that must be overcome to achieve clinical utility of TE. Full commitment to each germ layer in culture will be an important achievement in the development of tissue-engineered products containing stem cells. This can be achieved by optimizing growth factor combinations and use of advanced bioreactors, such as the one exemplified by scaled-up production of definitive endoderm in recent years [264]. Recent efforts to derive a wide variety of specialized cells from human ES and iPS cells are becoming successful. Examples include cardiomyocytes and endothelial cells, hepatocytes, insulin-producing beta-like cells, neuronal and other cells of the nervous system, including oligodendrocytes and subsets of astrocytes.

Embryonic stem cells

More efforts are needed for ensuring success of pluripotent stem cells (such as ES cells) as a source for cell therapy and tissue-engineered products. Purity of differentiated cell populations and exclusion of potentially tumorigenic undifferentiated stem cells must be confirmed for every application. In addition, the maturity of differentiated cells is still an issue, where some of the differentiated cells can retain embryonic phenotypes. For examples, detailed studies of various differentiated cell lineages (such as hepatocytes and pancreatic β cells) have highlighted the difficulty of achieving fully adult phenotypes from ES cells [265]. The completion of development may actually require additional maturation for several months in vivo, as shown for endocrine pancreatic progenitors of insulin-producing β-like cells. Therefore while serving as useful models for human development, the immature cells derived from pluripotent stem cells may have significant limitations as components of safe and effective tissue-engineered therapies.

In the future, a selection of small molecules could replace growth factors and hormones in driving key steps in the differentiation sequence and should facilitate development of cost-effective therapeutic products [266]. The production of ES cells and differentiation to pancreatic progenitors have been recently scaled-up under conditions compatible with GMP to support clinical testing [266]. It is anticipated that the grafted cells will be susceptible to acute immune attack, both by alloreactive T cells and by the autoimmune T cells that initially caused the patients' type 1 diabetes. One strategy to avoid this would be to deliver a tissue-engineered product with the therapeutic cells encapsulated in a semipermeable device designed to protect them from the immune system, while allowing exchange of nutrients and release of insulin to the circulation.

The need to protect grafts from the recipient's immune system is a fundamental problem for cell therapy and TE [267]. Due to the genetic mismatch between donors and recipients, the risk of immune rejection must be considered for any cell therapy or tissue-engineered products based on ES cells. One potential way to ensure successful allotransplantation of these cells would be to modify ES cells genetically to knock out MHC Class I, and possibly Class II, expression [267]. This might be the reason why differentiated ES cell derivatives show lower immunogenicity than do corresponding adult human cells [268]. There are some methods that can be used to induce immunological tolerance in recipients of allogeneic stem-cell-derived grafts [269,270]. One such method is nonablative treatment of the host with monoclonal antibodies to the T-cell antigens CD4 and CD8 [271] successful; this approach would not only allow for off-the-shelf also tissue therapies but also lower the safety and economic barriers to widespread adoption of ES cell-based therapies in medical practice. Another approach in this direction rests on cell banking strategies. The simple process of deriving clinically relevant ES cells from blood group O donors eliminates the issues of ABO type compatibility [267]. In addition, establishment of GMP-grade ES cell line banks

of relatively modest size (say 50−200 lines from appropriately selected donors) would enable complete and efficient matching at the most important MHC loci (HLA-A, -B, and -DR) for the large majority of the population. The derivation of ES cells from parthenogenetic embryos (that are homozygous for HLA loci) or selection of rare HLA-homozygous donors from the general population for obtaining pluripotent stem-cell lines can also significantly decrease the size of bank required to ensure matching of most potential recipients [272]. Stem-cell line banking on a global scale seems an idealistic but potentially feasible goal for future development of cell and tissue-engineered therapies [273].

Induced pluripotent stem cells

iPSCs are strong candidate cells for the future of TE. Already the next generation of reprograming methods are evolving where drug-like compounds can substitute for some of the transcription factors [274,275]. Indeed, recent studies have shown that efficient generation of iPSCs can be achieved using a single transcription factor *OCT4* and a cocktail of small molecules [276]. Another recent method utilizes a microRNA (miR-302) that regulates chromatin demethylation and other factors involved in global reprograming [277]. For future clinical application, it will be necessary that reprograming to generate iPSCs be carried out under GMP-compliant conditions. This has been accomplished to some extent [278]. Scale-up to yield clinical grade cell therapy and tissue-engineered constructs from iPSCs also raises the same essential manufacturing and regulatory concerns that have been discussed for the ES cells.

In spite of all the progress, questions still remain about whether iPSCs are completely equivalent to ES cells in their genetic makeup and in their potential to yield a full range of differentiated cell types. Critical issues that must be addressed for future therapeutic applications would center on the epigenetic mechanisms underlying the resetting of pluripotency [279,280]. Also to be addressed would be genetic and epigenetic variations that might persist through the reprograming process into the mature, specialized cells that will be used for therapy [281]. On the manufacturing side, it is possible that best practices from other cell therapy development will be applied to iPSCs and standards will be established to meet high level of uniformity and quality needed for such therapies. On the regulatory side, assurance of long-term performance and safety will be necessary, similar to what is required for any cell and tissue-engineered therapies.

Another future direction for iPSCs would be to use blood cells, such as the peripheral blood mononuclear cells, as preferred starting material for the production of clinical grade iPS cell lines [282]. The epigenetic and gene expression signatures of peripheral blood mononuclear cells have been shown to more closely resemble those of pluripotent stem cells, than for the fibroblasts. As for the cellular reprograming factors, the addition of Zscan4 (a zinc finger protein that is expressed selectively in 2-cell embryos) to the "Yamanaka" set of transcription factors decreased the DNA damage response during reprograming of mouse somatic cells and improved the yield of pluripotent cells [283]. This will be an interesting direction to explore for future use of iPSCs.

Adult stem cells

A relatively new cell source canned amniotic fluid-derived stem (AFS) cells that possibly represent a primitive precursors of MSC, have recently gaining a lot of attention. AFS cells are isolated from amniotic fluid or chorionic villi and share some properties with adult MSC but are capable of more extensive proliferation in cultures [284]. Since they also express some markers in common with ES cells, their precise lineage and physiological plasticity are still not certain. However, such as MSCs, AFS cells also possess immunomodulatory properties that could benefit their clinical utility [285]. A clear advantage over MSCs is their abundance (in amniotic fluid) and ease of isolation. For the treatment of neurodegenerative conditions and for neural TE, the brain-derived neural stem cells (such as HuCNS-SC) look promising. Future clinical trials based on HpSC may use cells sourced from neonatal livers that can be obtained through organ donation. Hepatic stem and progenitor cells are known to survive relatively long under ischemic conditions as compared to other stem cells [286]. Plus the development of cryopreservation and large-scale culture conditions for these cells will further make them popular for cell and tissue-engineered therapies in the future.

For liver TE, using HpSC in combination with endothelial progenitors such as the liver-derived angioblasts on whole-organ decellularized scaffolds can be a practical strategy [95,287]. Also delivering HpSC in a liver-specific matrix components (hydrogel) along with growth factors might help promote a more rapid, robust differentiation to hepatocytes [95]. In vivo studies in mice have confirmed that human biliary tree stem cells are multipotent for liver and pancreas lineages [288]. More studies will be needed with higher animal models and humans before these cells are declared useful for clinical applications. The biliary tree stem cells can easily be harvested from tissue that is frequently discarded after certain surgical procedures. Their easy availability and isolation (even through a simple procedure such as laparoscopy) represents a great value as a potential cell source for TE of islet-like structures for treating insulin-dependent diabetes.

Whole organ engineering

Use of decellularized organs is a practical approach of bioengineering whole organs for possible clinical use in transplantation. Studies of decellularized tissues and organs are advancing in scope, and some are already used in clinical studies. Since the ECM is much more than just a scaffold for cells and can also control cell organization, communication, and tissue functions, properties of the decellularized tissues can be a primary determinant and dictate reconstruction outcomes. New approaches in this area are focusing on developing decellularized tissues and organs with added benefits, such as retention of intact vasculatures, functionalization via biomaterials, and bioactive factors. When using decellularized tissues and organs for bioengineering new ones for transplantation, a complete assessment of decellularization is important. To prevent immune rejection, native antigens must be removed from the decellularized scaffolds. Also, of particular concern is the hyperacute immune rejection of a decellularized scaffold, which can occur anytime during the days or weeks after implantation [289]. This is caused by circulating antibodies to scaffold antigens within the host. Examples of decellularized scaffold that can trigger a hyperacute immune response include alpha-gal epitopes [289], structural proteins such as collagen VI of the ECM [290]. Even though structural proteins such as collagen VI are necessary for the mechanical stability of the scaffold, it is possible to limit their immunogenic effect. This will be necessary to prevent rejection after implantation into a host.

Every decellularization strategy can produce different effects on structural proteins within a scaffold. Therefore suitable method(s) must be chosen taking into consideration the target tissue type, tissue biomechanics, recellularization method, etc. Some commonly used chemical and mechanical decellularization methods have been known to damage ECM proteins [291]. Since it may be difficult to have a universal decellularization method for all tissue types, reagents and methods can be optimized based on tissue type, size, recellularization method being used, etc. The recellularization of the decellularized scaffold must also be optimized to produce a functional tissue or organ. The recellularization process has its own challenges. Primary among them is even distributions of the cells throughout the scaffold. Perfusion of cells through the vasculature of the construct is a common method used. Other methods include directly injecting cells into the tissue, using sonication to facilitate cell seeding and reendothelializing the vasculature of decellularized scaffold to improve seeding and organization of the cells [292]. Use of bioreactors has vastly improved the seeding and support of cells within decellularized scaffold for TE. For example, use of rotating wall vessel bioreactor has been shown to facilitated greater cell proliferation and viability [217]. Although great advancements have been made in whole organ bioengineering using decellularization, further efforts are needed to optimize reagents, methods, and equipment to achieve reproducible results. Use of a combined strategy that includes chemicals, mechanicals, and enzymes in a dynamic system may improve the decellularization efficiency and still retain components that will support efficient recellularization. For example, cells can be lysed using supercritical CO_2, followed by treatment with a low concentration of surfactants. Remaining cellular and genetic materials can be removed using wash with an enzymatic mixture, followed by buffers to remove all debris. Another area that needs further improvement is the scale-up of these decellularization and recellularization methods, so that enough scaffolds are available for making neo-tissues and organs to bridge the gap between donors and needy patients. Cell sourcing is also a bottleneck for whole organ bioengineering. With exploration of more adult stem-cell sources and human iPSCs, it would be possible to manufacture off-the-shelf tissues and organs in the future. This will help reduce or possibly eliminate the ever-increasing wait list and time for transplants.

Biofabrication technologies

Many TE applications have benefitted from the use of electrospun scaffolds. The high tunability of this technology can help both mechanical and bioactivity properties of the scaffolds. Recent advances in elecrospinning methods are further advancing these potentials by creating additional tools to guide cellular response and achieve enhanced tissue regeneration by generating aligned fibers, enhanced porosity, gradients of functional moieties, etc. New electrospinning methods such as coaxial electrospinning, fiber blending, emulsion electrospinning, and edge electrospinning can enable combining of different materials properties into a single scaffold. Other new methods such as coelectrospinning, hydrospinning, and 3D electrospinning are being used for generating complex fibers and scaffold architectures, and more developments are expected in the future. Enhancing cellular infiltration within electrospun scaffolds has always been a challenge. However, new electrospinning methods such as coelectrospinning, postelectrospinning processing and coaxial electrospinning have made it possible to ensure better and more uniform distribution of cells within electrospun scaffolds. Moving forwards, there are some common challenges to be addressed before electrospun scaffolds and tissues generated using them become mainstream in clinical applications. First, fiber formation, morphology, and composition have to be controlled, so that they are precise and reproducible in a manufacturing setting. Scaffolds with clinically relevant dimensions need to be made with sufficient process control of the electrospinning scale-up.

Cellular infiltration can be enhanced by further refining techniques such as multilayered electrospinning, cell layering, cell electrospraying, and dynamic cell culture systems. Despite these challenges, electrospinning remains a versatile technology and when combined with ever increasing innovative approaches, it is sure to have a lasting impact in the field of TE

Advances in 3D bioprinting hardware and the bioprinting processes are making it possible to fabricate tissues with different levels of complexities. However, this can present additional challenges when these technologies move from lab to commercialization. Accuracy and repeatability are two basic standards of quality that should be taken into consideration during manufacturing. Limiting the intrinsic variability during production can help reduce the extrinsic variability of such complex engineered tissue and organs when placed in in vivo environments [293]. Interestingly, 3D bioprinting, just like other additive manufacturing processes, can allow for in-process inspection of the product being produced, which is a positive attribute for quality control.

To increase the printing speed of multimaterial and multicell constructs, new 3D printing devices and strategies are being proposed. Also, development of perfusable vascular networks within biofabricated structures is also gaining importance from a clinical perspective. 3D cell culture techniques are also a recent trend and have contributed to significant progress in development of tissue-specific organoids, which is one of the scientific breakthroughs of recent years. Taken together, advances in 3D bioprinting, new bioink hydrogels, and 3D cell culturing are providing new opportunities for successful translation of tissue regeneration technologies and also gaining a better understanding of mechanisms involved in disease pathogenesis and progression. A future area of development in TE being made possible due to nanotechnology is referred to as "4D bioprinting." Here "time" is included as a fourth dimension in 3D bioprinting constructs, which can change their shapes or functionalities with time in response to external stimuli [118]. All these examples demonstrate the value of using nanotechnology in RM, which can add unique functionalities and broaden the portfolio of tools available for TE. Looking ahead, 3D bioprinting will benefit from advances in CAD/computer-aided manufacturing, standardization of materials, cells, and procedures to 1 day offer patient-specific tissue and organ repair therapies. There is no doubt that 3D bioprinting is transforming from an emerging trend to a main stream biomedical technology.

Tissue neovasculatization

3D bioprinting of vascular networks represents the best approach for creating vascular networks in engineered tissue and organs. Future efforts in this direction should also include design considerations, such as ability to withstand burst pressure and suitability for direct surgical anastomosis to host vasculature. The fabrication process can become even more complex when vasculature becomes part of the design. However, in vivo success of thick tissues and organs cannot be guaranteed without this.

Bioreactors

The type and complexity of the tissue being cultured in the bioreactor will affect the process design. Viscosity of the medium can also impose a mechanical load and affect cell morphology through cellular signaling [294]. It's been also observed that stem cells, when subjected to mechanical stimuli, can undergo what is known as mechano-differentiation [295,296]. All these topics are focus of intense research, which are going to influence future bioreactor designs. It is clear that to mimic in vivo organ function, there is considerable complexity required in bioreactor design. Use of computational modeling (also referred to as in silico analysis) using parameters from normal tissue/organ physiology and incorporating it into bioreactor construction can definitely improve TE outcomes. An example of this approach is a study where necessary parameters for vascular TE were derived by using a computer model to analyze fluid-structure interaction [297]. Results from this modeling matched results from an analytical model where predictions were made for pressures on the tubular scaffolds, wall shear stress, and circumferential deformation. Hence, use of in silico analysis can provide critical information that can enhance the development of the right bioreactor and culture conditions for complex tissues and organs.

Integration of nanotechnology

Current methods of cell patterning includes creation of physical patterns (using techniques such as ablation, etching, lithography, and printing), chemically modifying surface, or producing substrates with inherent topographies (fibers, hydrogels, etc.). All these methods have limitations of their own. Use of MNPs for cell control and patterning provides a new tool for fabricating tissue architecture that can match in vivo conditions. Examples of this approach include a study where a heterotypic coculture of mouse myoblast and human keratinocyte (HaCaT) cells was generated and capillary-like structures were formed with HUVEC cells [298]. Thus using nanoparticles, the biological properties of scaffolds and the resulting constructs can be significantly altered; thereby helping to engineering tissues with better overall function. Further integration and use of sensors and other electronic

elements in TE systems can support long-term studies and provide insights into the function, pathophysiological conditions, and also response to treatments. In a clinical setting, such system can monitor implanted tissues and organs in patients and provide important information on time if an intervention, drug adjustment, or other type of care is needed for the success of the implant. When translating nano-specific technologies to clinical applications, there are many challenges to overcome. For example, in applications where the chronic bioaccumulation of nanoparticles is a possibility, more long-term studies will be needed to determine safety. In products the notification of chemicals, criteria for classification, and labeling requirements are not widely available and will have to be revised. In addition to the technology itself, regulatory aspects will have to be addressed. Currently, there are no specific standards for nano-specific risk assessments within biological systems, nor clear regulatory guidelines for biological products containing nanoparticles. All these are challenges that need to be addressed for integration of nanotechnology in tissue-engineered clinical products.

Conclusions and future challenges

With advances in TE, technological advances such as use of stem cell, gene editing, 3D bioprinting, and nanotechnology, are enabling fabrication of tissue/organ with increasing complexities, resolution, and functionality, thereby supporting regenerative therapies that are tailored to specific patients and their needs. Use of electronic components or magnetic responsive elements within TE products can enable such features, where sensing and actuation can be controlled remotely and host response can be actively modulated postimplantation of the TE product. This has a potential to revolutionize not only patient follow-up but also regenerative therapies for human patients. Based on the requirements of individual patients, drug dosages, cells delivery, or treatment outcomes can be controlled which can significantly broaden the therapeutic efficacy of current TE systems. In addition, use of use of real-time monitoring and control systems in TE can advance our knowledge of disease mechanisms and homeostasis. One more area that can immensely help personalization of TE is use of artificial intelligence (AI) and large-scale data analysis. AI and data analytics can significantly accelerate design, fabrication, and testing of TE systems in future based on feedbacks from current products and processes. In all, use of personalized TE therapies can not only benefit patient outcomes but can also result in huge savings of social and healthcare costs, as inefficient and failed therapies are associated with huge costs at the healthcare and personal levels.

One of the future challenges in TE will be to develop technologies for faster biofabrication of complex, hierarchical tissue architectures, and human-scale dimensions for accurately recapitulating living tissues and organs. For successful TE, a smart biomaterial should not only mimic the structural components of the ECM but also be able to interact with cells in a dynamic way to promote adhesion, proliferation, differentiation, and tissue morphogenesis. However, the design of smart materials combines several of these properties is still a challenge. For example, it can be challenging to develop a smart material with optimal chemical and mechanical properties without negatively affecting its biological properties or vice versa. Of course, understanding the fundamental biology of tissue formation, growth, and maturation will be critical for developing bio-inspired materials, but deriving ideally fabricated structures out of them can further be challenging. This is due to the fact that TE by itself is a multistep, multicomponent approach, and finding the perfect solution at every step can be more difficult than easy.

Many novel synthetic and smart biomaterials have been developed in the last decade, and this trend is expected to continue. For biomaterials to be used for fabricating medical devices and tissue-engineered constructs, the development of efficient and safe sterilization, packaging, and storage methods, with minimal impact on the properties and performance of the material components will be necessary. Unfortunately, not much efforts are being places in this direction, which has to change if commercialization and clinical use of tissue-engineered products will have to be accelerated. Significant challenges for translation of biomaterial technology to the clinic still remain. A major limitation toward designing biomaterials for different TE applications is the limited fundamental understanding of the interactions between material and cells within tissues. Even though biomaterial engineering is producing materials with the ability to control cellular behavior to some extent, many challenges still remain in creating smart biomaterials that can respond to cells and their environments after implantation in vivo. Another challenge for the future would be the real-time monitoring and/or remote manipulation of tissue-engineered systems. Success in this direction will help establish a new paradigm for RM broadly.

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Further reading

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Chapter 2

From mathematical modeling and machine learning to clinical reality

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Introduction

Tissue engineering aims to understand the principles of in vivo tissue growth and development and applies this understanding to the engineering of functional replacement tissue ex vivo for clinical use subsequent to disease or injury. However, tissue formation is an inherently complex process—involving multiple biological, chemical, and physical processes that interact on a variety of length scales and timescales—and deciphering general principles of tissue development is a challenging task [1]. Nevertheless, a deep understanding of these principles is necessary to efficiently engineer functional tissue in vitro. For this reason, tissue engineering approaches commonly coordinate techniques from a variety of different disciplines, including molecular, cell and developmental biology, chemical engineering, and biophysics, to dissect this complexity. Recent years have seen an increasing interest in applying tools from the mathematical and computational sciences to the design, development, and implementation of tissue engineering protocols [2,3].

Mathematical and computational models are routinely used to help understand complex systems and optimize industrial processes in the engineering and physical sciences. There are many remarkable examples of the long-standing use of quantitative methods in the cell and molecular biosciences [4]. Recent rapid advances in both available computational power and high-throughput experimental procedures have stimulated an extraordinary cross-fertilization of ideas from the mathematical, statistical, physical, and life sciences, particularly within the areas of systems biology and bioinformatics [5]. Concurrently, much progress has also been made in recent years toward understanding general principles of tissue growth and development. In this chapter, we will explore how mathematical models, in collaboration with experimental studies and machine learning, can help further understanding of these principles. Given the breadth and rapid growth of this field, we will necessarily only focus on a few key areas, particularly: understanding molecular basis of stem and progenitor cell fate commitment; spatiotemporal regulation of cellular differentiation; and general principles of tissue morphogenesis.

Modeling stem cell dynamics

Stem cells are present during all stages of the development and are uniquely characterized by their capacity to maintain their numbers by self-renewing, as well as differentiate along distinct lineages. Embryonic stem (ES) cells have the capacity to produce all somatic tissues, a property known as pluripotency, and there is much interest in elucidating the molecular mechanisms of pluripotency [6]. Following the seminal work of the Yamanaka group in demonstrating that adult fibroblasts could be reprogramed to ES cell-like state by the forced expression of just four pluripotency-associated transcription factors (Klf4, c-Myc, Oct-3/4, and Sox2), there is a hope of using adult somatic cell−derived induced pluripotent stem (iPS) cells as patient specific, and less controversial, alternatives to human ES cells [7−9].

In contrast to ES and iPS cells, adult stem cells are typically lineage restricted and thereby constrained in their therapeutic potential (they are generally only multi- or unipotent) [10]. Nevertheless, due to their regenerative

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capacities and the relative ease, with which they may be obtained, there is also considerable interest in understanding the processes, by which adult stem cells orchestrate tissue growth and development and applying this understanding to engineering functional replacement tissue in the laboratory [1]. A standard tissue engineering approach to this problem is to harvest appropriate stem cell populations and, using defined culture conditions (which include regulating the chemical, physical/mechanical, and geometric properties of the growth environment), recapitulate in vivo regenerative processes to produce functional tissue in vitro. The ultimate success of this strategy relies on a clear understanding of the molecular mechanisms of stem cell self-renewal and differentiation and the spatiotemporal control of these processes.

Stem cell fate is critically dependent upon interactions with neighboring cells—including differentiated progeny [11] and other stem/progenitor cells [12]—and extracellular components that constitute the stem cell niche [13]. Stem cell interactions with the niche are mediated through intracellular molecular regulatory networks that include transcriptional, signaling, and epigenetic regulatory components [14]. These networks are highly complex, containing multiple feedback loops and feed forward loops that allow the cell to respond dynamically to changes in its environment. These types of feedback-based mechanisms are reminiscent of control systems in engineering, and recent years have seen considerable interest in viewing cell fate decisions from a dynamical systems perspective [14]. In the field of synthetic biology field, for instance, switches and oscillators have been implemented experimentally in synthetic model systems using rational designs based upon mathematical reasoning [15–17]. A general principle that emerges from this viewpoint is that the molecular switches that underpin stem cell fate decisions are ultimately dependent upon positive feedback loops in the cells' underlying intracellular molecular regulatory circuitry [18].

Positive feedback–based molecular switches

Positive feedback–based switches play a central role during the development by initiating all-or-none commitment events in response to external stimuli and commonly underpin stem and progenitor cell fate decisions, including during hematopoiesis [19]; neural development [20]; and human embryonic and mesenchymal and stem cell fate commitment [21,22]. In order to illustrate the general principle of a feedback-based switch, consider as an example the following dimensionless equations, which describe a simple "toggle switch" consisting two cross-inhibitory transcription factors, X and Y [15]:

$$\frac{dx}{dt} = \frac{\alpha_1}{1 + y^{\beta_1}} - x \tag{2.1}$$

$$\frac{dy}{dt} = \frac{\alpha_2}{1 + x^{\beta_2}} - y \tag{2.2}$$

where x and y denote the intracellular concentration of X and Y. Eqs. (2.1) and (2.2) describe the rate of change in expression of X and Y over time and state that X and Y are synthesized with effective rates α_1 and α_2, respectively (first terms on the RHS) and decay with constant half-lives (second terms on RHS). The parameters β_1 and β_2 capture cooperativity in repression, allowing for repression by multiprotein complexes and/or binding of multiple regulatory sites on the respective promoters, for instance. Note that although this system is characterized by repressive interactions, it nevertheless represents a simple positive feedback loop since both factors indirectly activate their own expression via inhibiting expression of the other. This kind of switch has been engineered in both *Escherichia coli* [15] and mammalian cells [23] and behaves as a genetic "memory unit" [15] (indeed it behaves similarly to an SR flip-flop for memory storage in electronic systems).

The dynamics of this system may be explored by plotting changes in expression of X and Y against each other to form the system's *phase plane*. The phase plane for this system is shown in Fig. 2.1A–C for different parameter values. For appropriate regimes, this system supports stable expression of *either X or Y*, but not coexpression of both, as one might expect (Fig. 2.1A and C). For instance, if the effective rate of synthesis of Y is higher than that of X, then Y is expressed, while X is repressed (Fig. 2.1A); by contrast, if the effective rate of synthesis of X is higher than that of Y, then X is expressed, while Y is repressed (Fig. 2.1C). In these cases, since only one stable equilibrium point is present, the system is said to be *monostable*. In general, monostability occurs in the absence of cooperativity ($\beta_1 = \beta_2 = 1$) or if the effective rates of synthesis of the two repressors are unbalanced ($\alpha_1 \gg \alpha_2$ or $\alpha_1 \ll \alpha_2$). However, if the effective rates of synthesis of the two repressors are balanced ($\alpha_1 \approx \alpha_2$) and transcriptional repression is cooperative ($\beta_1, \beta_2 > 1$), then these two alternate states may stably coexist (Fig. 2.1B). In this case the system is said to be *bistable* and it is the capacity of this system to possess different equilibrium patterns of expression depending on parameter values that allow it to act as a switch. Particularly, this system is a *toggle* switch, since expression of either of the repressors may be toggled "on" and "off" by transiently inducing changes in expression by varying the effective rates of synthesis α_1 and α_2 [15].

Ultimately, the toggle switch described earlier works, since each factor directly represses transcription of the other while indirectly activating its own transcription.

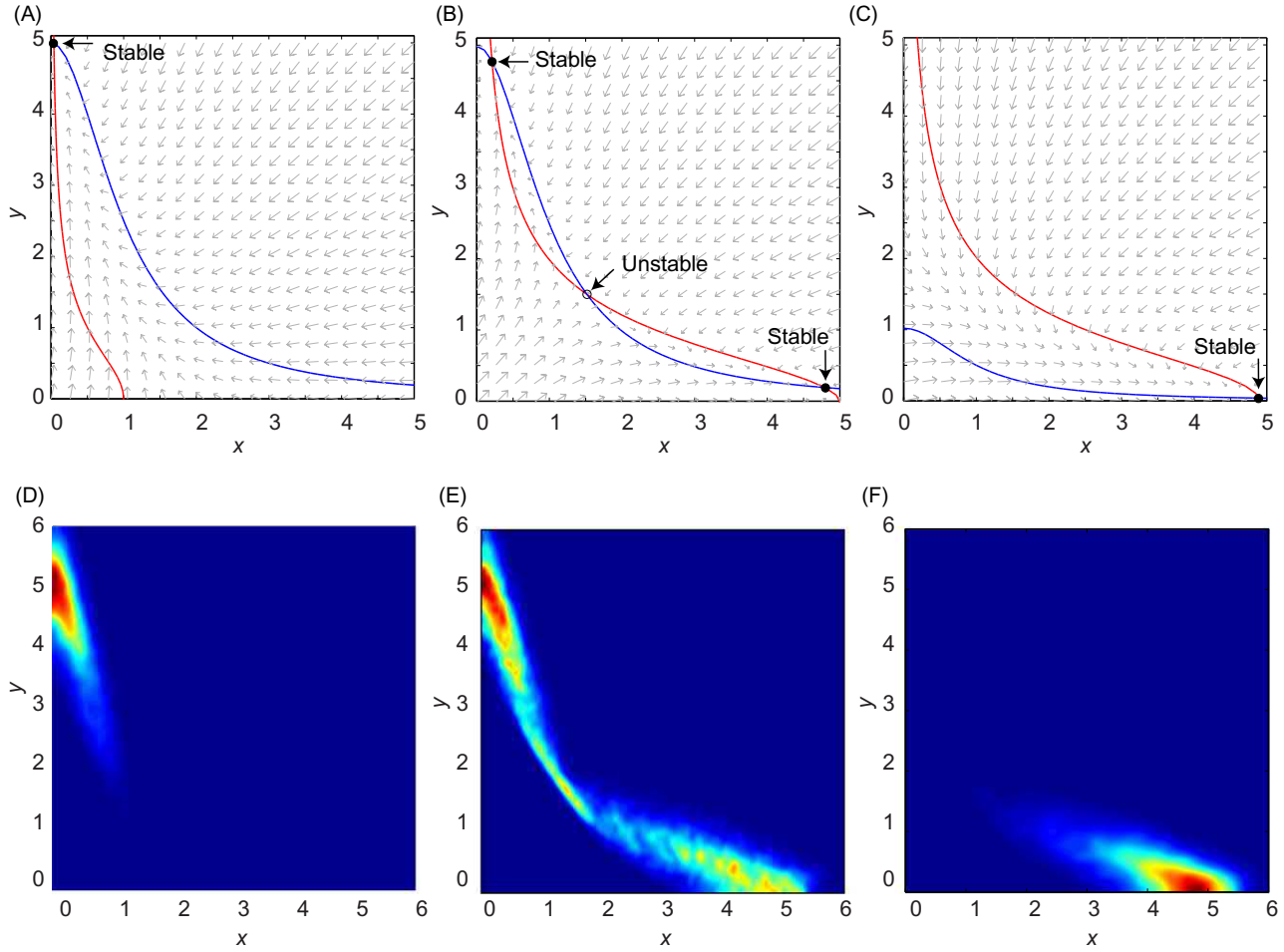

FIGURE 2.1 **Bistability and monostability in a genetic toggle switch.** (A–C) Phase planes for Eqs. (2.1) and (2.2) for different model parameters showing that this simple molecular switch supports both monostability (A and C) and bistability (B). Arrows show the direction field illustrating the flow toward the stable equilibria from different initial conditions. (D–F) Corresponding probability density functions show that expression variability can arise within a cellular population in the presence of molecular noise. Red shows regions of high probability of finding a cell; blue shows regions of low probability. Here stochasticity in expression of the two genes has been modeled as white noise process with amplitudes $\sigma_1 = 0.55$ (noise in expression of x) and $\sigma_2 = 0.75$ (noise in expression of y). Parameter values: (panels A and D) $\alpha_1 = 1$, $\alpha_2 = 5$; (panels B and E) $\alpha_1 = 5$, $\alpha_2 = 5$; (panels C and F) $\alpha_1 = 5$, $\alpha_2 = 1$. In all cases, $\beta_1 = \beta_2 = 2$.

However, mutual cross-repression is by no means the only positive feedback motif that can give rise to molecular switches of this kind. For instance, direct autoregulation, which is also common in eukaryotic genetic regulatory networks [16,24], can give rise to multistability and switching, either on its own [16] or in concert with other mechanisms. For example, if in addition to mutual cross-repression, each of the transcription factors in the toggle switch also directly regulates its own expression positively then a third "primed" state, in which both factors are promiscuously coexpressed within individual cells, may also be supported resulting in a potentially *tristable* system. This occurs, for instance, during hematopoiesis: the lineage-specifying master transcription factors, GATA1 and PU.1, each enhance their own expression while repressing that of the other [25]. Since both GATA1 and PU.1 also regulate multiple downstream targets (GATA1 specifies the erythroid/megakaryocyte lineages, while PU.1 specifies the myelomonocytic lineage), the state of this motif affects widespread genetic programs and effectively defines a lineage choice. By differentially regulating multiple downstream targets, positive feedback–based switches, such as this, can act as input/output (I/O) devices within larger genetic regulatory networks [26]. In the context of development, positive feedback–based I/O devices are important, since they provide a mechanism by which the cell populations can convert spatial information (for instance, an extracellular morphogen gradient) into a defined commitment responses, resulting in spatial localization of widespread gene or protein expression patterns [16,27]. We will discuss further how spatial localization of intracellular feedback mechanisms

can give rise to complex spatiotemporal patterns of expression in the section "Pattern formation."

Variability in stem cell populations

Although useful, the toggle-switch model given in Eqs. (2.1) and (2.2) is deterministic, and it does not account for environmental fluctuations or variability in gene expression, which are inevitably present. Gene and protein expression are intrinsically stochastic processes [28,29] and cell−cell variability may arise in isogenic cell populations either due to gene expression "noise," or due to stochastic partitioning errors or asymmetry during cell division [30−32] (Fig. 2.2). For instance, if such expression noise is accounted for in the toggle-switch model, then cell−cell variability in expression patterns can naturally arise within a population of genetically identical cells (Fig. 2.1D−F). This variability is particularly evident in the bistable regime, when expression fluctuations can induce transitions between the coexisting stable states (Fig. 2.1E). This kind of cell division−independent, nongenetic population heterogeneity is remarkably robust and appears to be a relatively widespread feature of both prokaryotic and eukaryotic cell populations [33−35].

It has long been postulated that stochasticity may have an important role in stem cell fate commitment [36−39]. In the 1960s Till et al. assessed the colony-forming ability of adult mouse bone marrow cells in the spleens of irradiated mice [36]. Using a serial transplantation assay, they found that the number of colony-forming units (CFUs) per colony varied considerably beyond that expected from sampling errors alone, in a manner consistent with a "birth-and-death" process (a simple Markov process, well studied in the statistical physics literature [40]), in which CFU self-renewal and differentiation occur stochastically with defined probabilities [36]. They confirmed this conclusion by numerically simulating colony formation using the birth-and-death model, providing a good early example of the use of computational simulations in stem cell biology. Similarly, Ogawa et al. came to related conclusions concerning the stochastic nature of stem cell fate commitment, in their studies of paired hematopoietic progenitor cells in the 1980s [37−39].

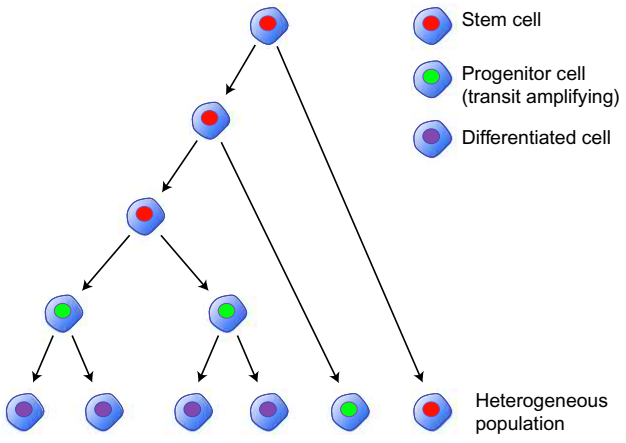

FIGURE 2.2 Heterogeneity in a hierarchically structured population of stem, progenitor, and terminally differentiated cells. Stem cells maintain their numbers by symmetric and asymmetric divisions and give rise to transit amplifying progenitor cells, which in turn produce terminally differentiated progeny. This hierarchy of divisions naturally produces a mixed population containing stem, progenitor, and terminally differentiated cell types in proportions that vary with environment, age, and disease or insult.

Within the last few years, studies have confirmed substantial variability in both equilibrium expression patterns in a variety of mammalian stem and progenitor cell populations [33,41−45] and the dynamics of cellular differentiation and reprograming [21,46,47]. In our own recent work, we have investigated variability in colony-forming ability of primary human bone marrow stromal cell (HBMSC) populations using a two-stage colony-forming assay [48]. We found considerable variability in both the sizes of primary (P0) colonies, seeded from single HBMSCs, and secondary (P1) colonies, formed by detaching cells from P0 colonies and reseeding at clonal density, with apparently little correlation between primary and secondary colony sizes (dependent on the primary colony, transplanted cells produced either small secondary colonies or a wide range of disparate colony sizes). We also observed distinct spatial heterogeneities in expression of mesenchymal and niche-associated markers (including CD146, alpha smooth muscle actin, and alkaline phosphatase, see Fig. 2.3A−C) indicating spatial regulation of cell behavior within the colonies. Computational analysis, using a cellular automaton model that accounted for proliferation and migration of individual cells, showed that this behavior is consistent with tissue growth within a structured cellular hierarchy (consisting stem, transit amplifying, and differentiated cell types, see Fig. 2.2), regulated by cell−cell interactions.

Taken together these results indicate that cell−cell variability in expression patterns and stochasticity in stem cell commitment may be closely related, with stochastic mechanisms allowing dynamic "priming" of subpopulations of stem cells for different lineages prior to commitment [49]. These observations suggest that cell fate commitment events are regulated at the population, rather than individual cell, level. Thus intracellular molecular regulatory networks may not control individual stem cell fate commitment per se, but rather control the overall structure of the cellular population [36], possibly by modulating molecular noise [50]. In this regard a more probabilistic view of stem cell fate may be appropriate, in which statistical properties of ensembles of cells,

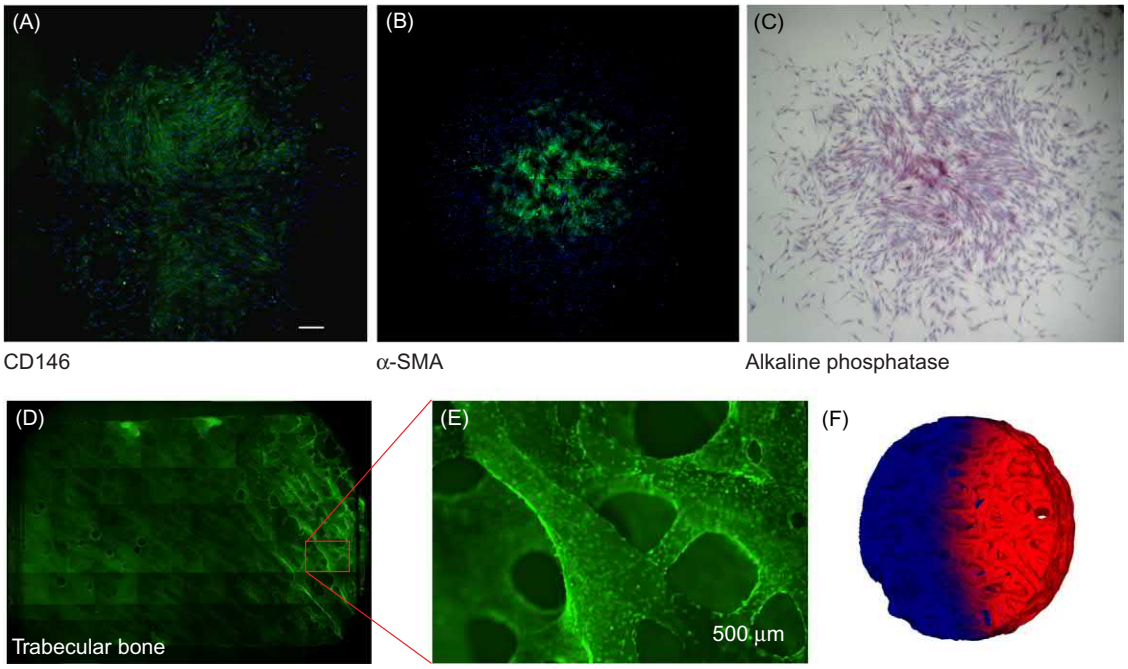

FIGURE 2.3 **Tissue growth in monolayer and 3D culture.** Panels (A–C) show intracolony variability of differentiation and niche markers within primary human bone marrow stromal cell samples seeded at clonal density to form single cell–derived colonies. P0 colonies were fixed and assessed for the following mesenchymal markers. (A) CD146 (melanoma cell adhesion molecule) expression measured using mouse monoclonal antibody, in combination with fluorescein isothiocyanate (FITC)-conjugated antimouse IgG secondary antibody. (B) Alpha smooth muscle actin expression measured using mouse monoclonal antibody in conjunction with FITC-conjugated antimouse IgG secondary antibody. (C) Alkaline phosphatase activity in red, stained with naphthol AS-MX phosphate and fast violet B salts. Staining of all three markers is strongest toward the center of the colony. Panels (D–F) show cell colonization of complex 3D geometries. (D) Slice of human trabecular bone used in a 3D colonization assay. (E) Primary human bone marrow stromal cells were seeded onto the trabecular bone sample and cell colonization was visualized over time using CellTracker Green labeling (bright green). (F) Typical simulation of corresponding mathematical model of cell proliferation and colonization of trabecular bone sample. Domain geometry was obtained from a μCT scan of the trabecular bone sample. Regions of high cell number density are in red; regions of low cell number density are in blue. *3D*, Three-dimensional; *IgG*, immunoglobulin G; *μCT*, microcomputerized tomography.

rather than characteristics of individual cells (or averaged properties of populations of cells) are considered. An enhanced quantitative understanding of the intrinsic variability of stem cell populations will require developments in both high-throughput single-cell profiling procedures and the mathematical and statistical techniques needed to decipher the complex datasets that result from these experimental advances. Such advances will enable a better understanding of how variability can be controlled to produce more defined stem cell populations, which will ultimately lead to the development of more robust stem cell isolation and expansion protocols for tissue engineering and regenerative medicine applications.

Modeling tissue growth and development

Any success in engineering functional tissue ex vivo must rely not only on deciphering individual stem cell behavior but also on developing an enhanced understanding of the mechanisms of morphogenesis in vivo, including the environmental, chemical, mechanical, genetic, and epigenetic processes, involved in spatial regulation of cellular differentiation. There have been a number of remarkable recent successes in engineering complex, spatially structured, and *functional* three-dimensional (3D) tissues in the lab, including the optic cup [51]; the anterior pituitary (adenohypophysis) [52]; the trachea [53]; articular cartilage [54,55]; vascularized bone [56]; and, recently, allogeneic veins [57].

The formation of structure either during in vivo development or ex vivo tissue formation is dependent upon cells translating spatial information concerning their relative location within the developing organism or tissue into different patterns of gene and protein expression based upon their current genetic state and developmental history [58]. The extraordinary reproducibility of development requires not only that positional information is precisely and robustly defined (i.e., insensitive to environmental fluctuations) but also that cellular sensing of such information is correspondingly sensitive and appropriately coordinated. Spatial gradients have long been known to provide positional information for spatial patterning during development (for instance, see the work of Wolpert and coworkers [59–61]). Since spatial patterns emerge on relatively

short length scales, Crick suggested that spatial information may be supplied by diffusion gradients [62], although local mechanisms, such as cell−cell communication, are also important [63]. Consequently, mathematical models of reaction−diffusion processes—which take into account both chemical diffusion and cellular responses to evolving chemical gradients—have been widely used to study tissue growth and development.

Monolayer tissue growth in vitro

One of the simplest reaction−diffusion models is Fisher's equation, which was originally used to study the spread of a favored gene through a population [64] but serves as a good model for expansion of an in vitro monolayer cell colony due to proliferation and cellular migration. Denoting the cell number density (the number of cells/m^2 for monolayer culture) at position $\boldsymbol{x}$ and time t by $n(\boldsymbol{x}, t)$, Fisher's equation reads the following:

$$\frac{\partial n}{\partial t} = rn(N - n) + D\nabla^2 n \tag{2.3}$$

This equation describes the rate of change of cell number density in space and time. The first term on the RHS of Eq. (2.3) describes cellular proliferation. At low cell number densities proliferation is approximately exponential, with linear growth rate rn; however, as the cell number density reaches N, the maximum at confluence, the proliferation rate tends to zero accounting for inhibition of cell division due to cellular crowding. This model is known as logistic growth and is a good first approximation to two-dimensional expansion of an unstructured population of identical cells. Hierarchically, structured populations that contain subpopulations of stem, progenitor, or transit amplifying cells may exhibit more complex patterns of proliferation not captured by the logistic model (Fig. 2.2). Mathematical models of growth kinetics in such heterogeneous populations have been considered by a number of authors [48,65−67]. The second term on the RHS of Eq. (2.3) describes cell motility and states that cells move in a random manner with motility coefficient D, similar to a diffusion coefficient. More complex forms of motility, including chemotaxis or haptotaxis, may be included by adapting this migration term [68]. In one spatial dimension, assuming appropriate initial conditions, Fisher's equation predicts that the colony advances as a "traveling wave" (meaning that the invasion front maintains a constant shape, moving at a constant speed) with wave speed $v = 2\sqrt{rND}$ [64]. In the case that growth is spherical and axisymmetric (for instance, a radially expanding colony), the wave speed approaches this constant asymptotically for large radius [64]. Since the wave speed depends upon both the diffusion coefficient and the linear growth rate, reaction−diffusion fronts may advance much faster than by diffusion alone, and in a precisely quantifiable way. Consequently, Fisher's equation provides an illustrative example of how a simple mathematical model may be used to investigate the relationship between proliferative and migratory parameters and colony growth rate. If the linear growth rate is known (for instance, from an independent proliferation assay), then cellular motility coefficients may be estimated using this relationship. For instance, Maini et al. have used this model to analyze an invasion front in a wound-healing assay for human peritoneal mesothelial cells [69,70] and we have previously used this relationship to estimate the migration rate of primary HBMSCs [71]. We found that for slowly proliferating migratory cell types, such as HBMSC populations, Fisher's equation provides a good model for colony growth; however, for highly proliferative populations, such as the MG63 human osteosarcoma cell line, a sharp-front variation of Fisher's model is more appropriate [71].

Tissue growth on complex surfaces in vitro

Fisher's equation as outlined earlier describes monolayer growth. However, tissue engineering strategies commonly rely on seeding appropriate cell populations onto complex 3D porous biomimetic scaffolds in order to provide geometric support for tissue development [1,72]. In this situation, while good peripheral tissue growth is usually observed, poor growth or necrotic cell death is often seen in the central regions of such constructs (though this is not always acknowledged). Spatial heterogeneity of this kind is usually related to inadequate nutrient supply and/or accumulation of toxic waste products at the scaffold interior, problems that we shall discuss in the following section. However, spatial tissue inhomogeneity may also arise from poor cellular ingrowth due to uneven initial seeding of cells and/or locally adverse scaffold geometries. Numerous groups have investigated the effects of seeding strategies, scaffold geometry, and nutrient supply on subsequent tissue growth, using both experimental and computational methods [73−75]. For instance, we have investigated HBMSC population ingrowth in a complex geometry using an experimental−computational approach, which simulated Fisher's equation over a surface obtained from microcomputerized tomography scanning of trabecular bone [75] and compared simulations with experimentally derived invasion fronts. We observed that despite the complex geometry, Fisher's equation described tissue ingrowth over the trabecular bone surface surprisingly well, with the average migration front advancing at an approximately constant speed dependent upon geometric properties of the bone surface, such as porosity and tortuosity [75] (see Fig. 2.3D−F). Applications of simple models of this kind are useful, since they allow quantitative

assessment of how scaffold architecture affects key proliferation and migratory parameters that govern the efficacy of tissue ingrowth.

Three-dimensional tissue growth in vitro

Nutrient-limited growth has been observed for a wide variety of engineered tissues, including articular cartilage, intervertebral fibrocartilage, and cardiac tissue [74,76,77]. In the absence of enhanced transport of nutrients and cellular waste products, spatial heterogeneities in cell numbers rapidly develop: peripheral cells have access to ample nutrient that they consume, leaving those on the interior nutrient starved. Consequently, diffusion-limited viable scaffold-based tissue growth is typically restricted to a few hundred micrometers (approximately the intercapillary distance in vivo). Spatial variability in cell numbers can also lead to variability in deposited extracellular matrix components that can severely inhibit the formation of mechanically competent tissue (see Fig. 2.4). The problem of nutrient-limited growth and resultant tissue integrity has been examined from a computational perspective by numerous groups in both the context of tissue engineering [74,78–80] and avascular solid tumor growth [81].

For example, we used the following mathematical model to investigate the relationship between evolving oxygen concentration profiles and nutrient-limited tissue formation in the early stages of growth in developing engineered cartilage tissue [74]:

$$\frac{\partial c}{\partial t} = D\nabla^2 c - \alpha\beta cn \tag{2.4}$$

$$\frac{\partial n}{\partial t} = \beta cn \tag{2.5}$$

where $c(\boldsymbol{x}, t)$ and $n(\boldsymbol{x}, t)$ represent oxygen concentration (mol/m^3) and chondrocyte cell number density (number of cells/m^3 for 3D culture) at position $\boldsymbol{x}$ and time t, respectively. These equations describe the rate of change of oxygen concentration and cell numbers in both space and time. The first one describes the evolution of the oxygen concentration in the developing tissue: the first term on the RHS of this equation accounts for transport of oxygen, which is assumed to occur by diffusion with constant diffusion coefficient D; the second term accounts for oxygen depletion and states that oxygen is consumed at a rate proportional to the local cell number density. The second equation describes changes in cell number density: the term on the RHS accounts for cellular proliferation at a rate proportional to the oxygen concentration (cell death and migration are assumed to be negligible during the initial phase of tissue growth, which these models consider). The parameters α and β control the relative rates of cellular proliferation and oxygen consumption. Note that the cell number density and the oxygen concentration are intrinsically coupled to each other: changes in oxygen

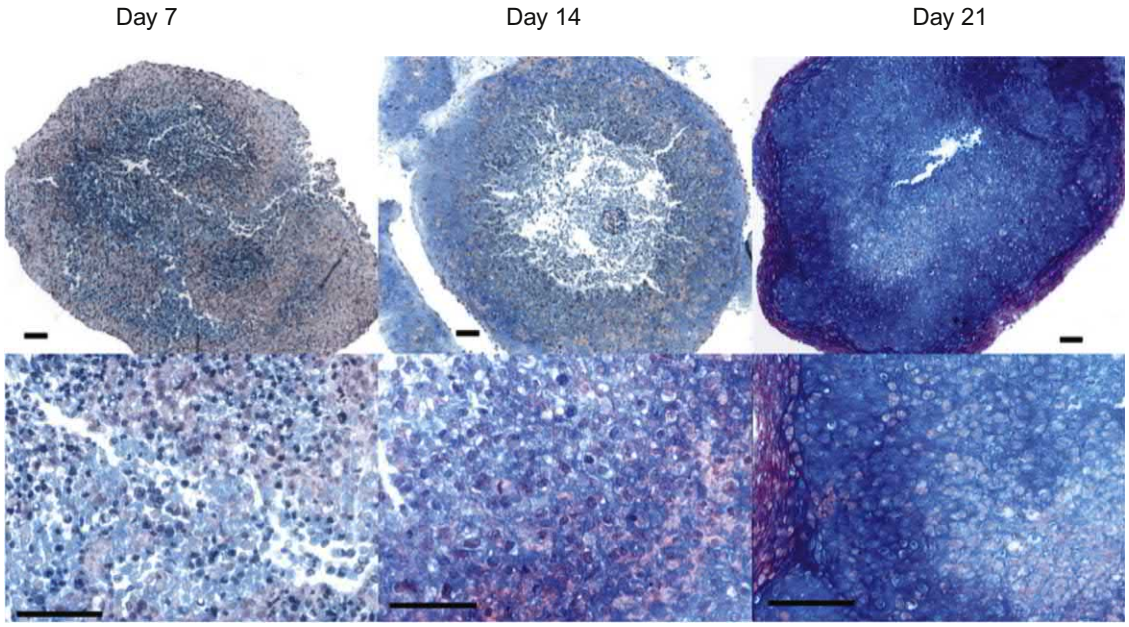

FIGURE 2.4 21-Day pellet culture time course of murine chondrocytic ATDC5 cells. Pellets were stained with alcian blue and sirius red to demonstrate the presence of proteoglycans and collagenous ECM, respectively. Spatial variability in cell numbers is apparent after 7 days and extends throughout the culture period. Cell number variability also leads to variability in deposited ECM components, which affects the structural properties of the engineered tissue. Scale bar: 100 μm. *ECM*, Extracellular matrix.

concentration affect cellular proliferation, which in turn effect changes in oxygen levels. To close the system, we assume that oxygen levels are initially constant and the scaffold is uniformly seeded with chondrocytes and take a no-flux boundary condition at the scaffold center and a continuity condition on the scaffold surface for the oxygen concentration. Although this model is somewhat simplified, it was nevertheless found to provide a good fit to experimentally derived evolving oxygen and cell number density profiles, indicating that oxygen availability is a rate-limiting process during the early stages of in vitro cartilaginous tissue formation. Furthermore, this analysis indicated that in the absence of enhanced transport processes or regulation of cellular proliferation, spatial heterogeneities are inevitable: nutrient supply by diffusion alone will always result in proliferation-dominated peripheral regions that severely restrict viable tissue growth, and therefore potential for clinical scale-up. This problem has been tackled both by enhancing nutrient transport, for instance using bioreactors, in which transport is enhanced by advection or perfusion [82,83], or using printed scaffolds that possess an artificial "vasculature," which can channel nutrients to the tissue center [84]; and by coculture with endothelial cells in order to encourage the concomitant formation of de novo vasculature within the developing tissue [85].

Pattern formation

During development, extraordinarily complex spatiotemporal structures emerge spontaneously, and it is these intricate "patterns" that ultimately provide tissues and organs with the microscopic and macroscopic structure necessary to their function. Reproducing such pattern-forming processes in vitro is a major goal of tissue engineering, and a quantitative understanding of these self-organizing processes will be needed to reproducibly and reliably engineer macroscopically structured functional tissues ex vivo. Remarkable progress has been made toward understanding the biochemical and biophysical basis of morphogenesis from both experimental and theoretical perspectives (see Ref. [86] and references therein for some examples).

In the 1950s Turing presented a simple chemical mechanism, by which spatial patterns may arise spontaneously in biological systems [87], which has become acknowledged as a milestone in our understanding of development [88]. Turing's mechanism relies upon diffusion of a chemical "morphogen" destabilizing a spatially homogeneous state, and driving evolution toward a stable spatially heterogeneous, or patterned, state. This mechanism is both simple and remarkable, since diffusion usually has a homogenizing, or stabilizing, role. However, it can give rise to an extraordinary range of complex patterns. Consequently, it has received much attention in the mathematical biology literature [89]. The biochemical and biophysical basis of morphogenesis has developed considerably, since Turing's initial work and his concepts have been used to help understand a range of mammalian developmental systems, including vertebrate limb bud development, angiogenesis, and wound healing [64,89,90]. Recent developments have also begun to take into account both chemical mechanisms and biomechanical forces (which are known to play a critical part in the development of tissues, such as bone [63]) in morphogenesis [91,92].

An archetypical model of pattern formation is the *activator–inhibitor* model of Gierer and Meinhardt [93]. The generalized Gierer–Meinhardt (GM) model consists of the following coupled equations, which describe reactions between an *activator* with concentration $u(\boldsymbol{x},t)$ (mol/m^3) and an antagonist, or *inhibitor*, with concentration $v(\boldsymbol{x},t)$ (mol/m^3) at position $\boldsymbol{x}$ and time t:

$$\frac{\partial u}{\partial t} = D_u\nabla^2 u + \rho_u + \frac{\rho u^2}{(1+\kappa u^2)v} - \mu_u u \tag{2.6}$$

$$\frac{\partial v}{\partial t} = D_v\nabla^2 v + \rho_v + \rho u^2 - \mu_v v \tag{2.7}$$

These equations describe the rate of change of activator and inhibitor concentrations in space and time. Here $D_u, D_v, \rho_u, \rho_v, \rho, \mu_u$, and μ_v are positive constants, which characterize the rates of diffusion, production, and decay of the two species. The first terms on the RHS of Eqs. (2.6) and (2.7) account for diffusion; the second and third terms account for production: the activator enhances its own production (forming an autocatalytic positive feedback loop) and that of the inhibitor, while the inhibitor represses production of the activator; the fourth terms account for the decay of both species with constant half-lives. If the inhibitor diffuses more quickly than the activator, then this model can give rise to a variety of different spatially inhomogeneous patterns (including various types of spots and strips, see Fig. 2.5 for some examples). The essential mechanism, by which these patterns arise, depends on spatial control of positive feedback: small fluctuations away from the spatially homogeneous state are initially strongly locally amplified by the positive feedback present in the system due to activator autocatalysis; however, this amplification does not continue uninhibited but rather is spatially confined due to fast diffusion of the inhibitor away from regions of local activation. This type of *short-range* activation and *long-range* inhibition underpins many examples of biological pattern formation. For instance, using a joint experimental–computational approach, Garfinkel et al. have recently shown that this mechanism can account for complex patterns formed by adult vascular mesenchymal

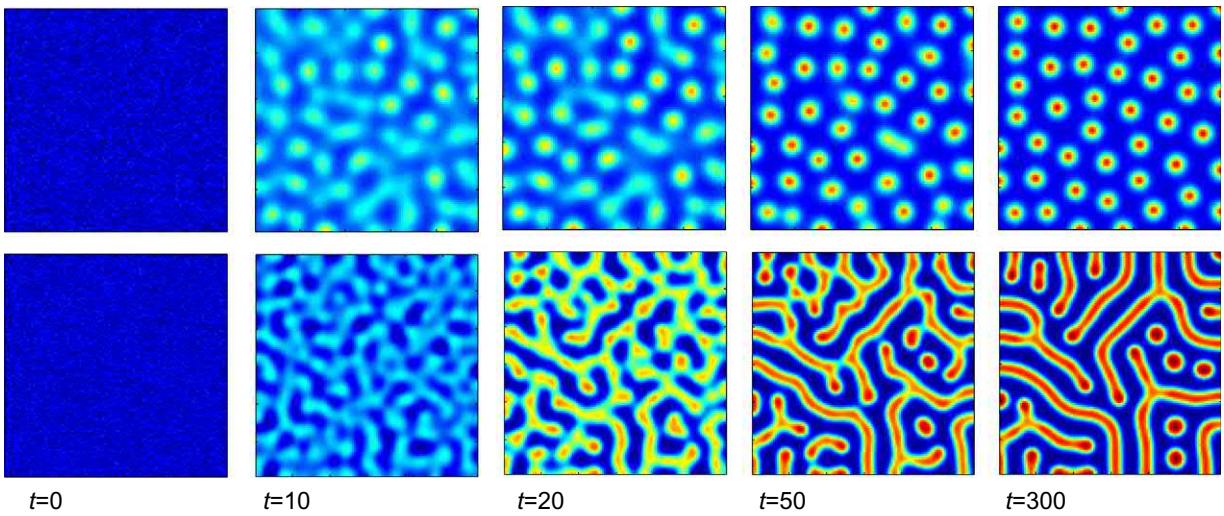

FIGURE 2.5 Spontaneous pattern formation in a simple reaction–diffusion model. Simulations of the generalized Gierer–Meinhardt model [93], a classic activator–inhibitor reaction–diffusion process given in Eqs. (2.6) and (2.7), show how different kinds of spatial patterns can emerge spontaneously from the same model equations with different parameters. This model has been used to explain experimentally observed patterns formed by adult vascular mesenchymal cells in culture [94]: (top row) emergence of regular spots and (bottom row) emergence of stripes.

cells (VMCs) in monolayer culture in vitro [94]. VMCs differentiate and self-organize into variety of complex spatial patterns, similar to those seen in the GM model, during development, disease progression, as well as in vitro culture. Garfinkel et al. reasoned that bone morphogenetic protein 2 (BMP-2), which is expressed by VMCs and positively regulates its own transcription, may act as a local activator; while matrix gamma-carboxyglutamic acid protein (MGP), a relatively small molecule that inhibits BMP-2 induced differentiation, may play the part of a fast diffusing long-range inhibitor (see Ref. [94] and references therein). They found that experimental culture of VMCs demonstrated good qualitative agreement with the patterns formed in a generalized GM model based on spatiotemporal interactions between BMP-2 and MGP. In addition, their analysis predicted that different patterns would emerge from the same underlying mechanism if the kinetics of BMP-2 activation and MGP inhibition were perturbed. To test this, they showed experimentally that addition of MGP to the media preparation shifted the observed pattern from labyrinthine stripes to spots, while addition of warfarin (which blocks MGP inhibition of BMP-2, see Ref. [94] and references therein) resulted in "stripe-doubling," two changes that were predicted by their mathematical model.

Despite the relative success of activator–inhibitor models, such as this, Turing-type patterns are difficult to engineer synthetically, since they are sensitive to parameter changes and rely on particular disparities in kinetic and transport parameters. Consequently, the importance of these mechanisms in development has been long debated [95]. However, Turing did not intend his model to be an accurate description of any particular morphogenetic process, nor did he intend it to make hard biological predictions. Rather, the power of his work was that it initiated a new way of thinking about how complex self-organizing patterns may arise when feedback is spatially controlled. Perhaps, the greatest advantage to the tissue engineer of mathematical models of this kind is that they provide examples of how simple chemical, biological, and physical processes can interact spontaneously to produce complex spatial patterns in ways that are hard to explore using experiment and intuition alone. In the long term, better understanding of the complex self-organizing mechanisms of tissue and organ morphogenesis from such a mathematical perspective may allow more efficient and reproducible engineering of macroscopically structured tissue.

Machine learning in tissue engineering

Recent years have seen significant advances in the development of sensitive biochemical assays that reveal previously inaccessible aspects of cell and tissue biology. A representative example of these advances is single-cell sequencing technologies, which now enables the (parallel) genome-wide quantification of transcript abundance, gene accessibility, epigenetic modifications, 3D genome organization at single-cell resolution, providing information on thousands of features from different intracellular information levels [96].

While mathematical models have been tremendously successful in understanding, in detail, the interactions of a limited number of genes and their products (see Refs. [97–99]

for some recent examples), obtaining and dissecting a full mathematical representation of large numbers of genes and proteins remains challenging, since mathematical models quickly become analytically intractable due to the large number of interacting parameters. Therefore rather than modeling data directly, tools from statistics and computer science are needed to extract predictive patterns from complex datasets, which may then be used to inform the development of mathematical models. The modeling task is thus partitioned into complementary phases consisting a statistical analyses or machine learning to reveal the structure in the data and to provide a simplified phenomenological description, followed by the generation of hypotheses of how observed structures may have emerged. The latter can subsequently be validated experimentally or translated into mathematical models to probe the basic biological principles that give rise to the observed phenomena, as described, for instance, in the previous sections of this chapter.

Supervised methods

Finding (or recognizing) patterns in high-dimensional data is a common problem in data analytics. *Supervised* machine learning methods address this task by "learning" how combinations of measured input features (e.g., gene expression levels) combine to elicit a given response (e.g., a desired cell function) that can be discrete (for classification) or continuous (for regression). If response variables are known (at least for a representative subset of observations, known as the training set), then the mapping from input features to responses can be learnt by comparing the discrepancy between true and predicted responses and choosing the mapping that minimizes this discrepancy. Once such an optimal mapping has been established, it can be used to predict responses for unseen data. The key challenge is therefore in finding such an optimal mapping. There are a number of different supervised methods that are widely used to address this task.

Support vector machines (SVMs) learn a (high-dimensional) decision boundary for binary classification tasks [100]. SVMs have been used in tissue engineering, for example, to correctly predict the toxicity of drugs on tissue-engineered brain organoids in vitro [101]. As another example, SVMs were able to positively identify mesodermal cells (expressing the protein *Brachyury*) using cell density and *myosin light chain* phosphorylation levels (as a proxy of local tissue tension) as input features [102].

Random forests (RFs) are ensembles of decision trees, which can be used for classification or regression [103]. RFs have been used to successfully explore a number of single-cell expression datasets, including to predict muscle stem cell quiescence from expression profiles [104]; interrogate the effect of thrombopoietin on megakaryocyte commitment in hematopoietic stem cells [105]; and identify the physical interactions between stem and niche cells in the bone marrow [106].

Increasingly, artificial neural networks (ANNs), particularly deep neural networks, are being used to address problems in regenerative medicine. In particular, in the field of image classification and object detection, a variant of ANNs called deep convolutional neural networks (CNNs) have achieved expert-level performance, for instance, matching the classification accuracy of dermatologists in detecting skin cancer [107]. The superior performance of deep CNNs stems from their ability to learn representations directly from data that independently extract important features, such as color, shape, and regularity, and encode specific structures, such as abnormal skin patterns, as a combination of learnt features. However, the complexity of deep ANNs (which can have several thousand to millions of free parameters that must all be learnt from the training data) necessitates much larger training datasets, typically containing >1 million labeled examples, which may be impractical for many applications.

Transfer learning presents a common solution to this problem. Here, a set of well-annotated but unrelated training data is used to learn generic features that are commonly associated with the input data type(s) yet not specific to the particular problem at hand. Once established, it has been shown that such pretrained models may be subsequently retrained on a much smaller number of labeled examples that are specific to the problem of interest. In the case of skin cancer detection, a model, trained to recognize 1000 completely unrelated object categories (e.g., cars and animals) using 1.28 million annotated example images, was retrained on 127,000 images of different types of skin disease associated with 757 skin different disease classes [107], thus significantly reducing the amount of data and time required for training. We predict that such approaches will increasingly become implemented in standard analysis pipelines—for instance, in image analysis of cells and tissues—and will significantly aid the development of new biological insights.

Unsupervised methods

In the case where responses are not known a priori, methods from *unsupervised* learning can help find structures in data by optimizing a loss function that relies entirely on intrinsic properties of the data itself. Clustering is an example of such an unsupervised method. For instance, in K-means clustering [108], data are split into a prespecified number of clusters (K), which are determined using a loss function that aims to assign cluster membership in a way that minimizes the intracluster variability while maximizing the intercluster variability. The simplicity of this

approach has led to its widespread application to complex data, such as single-cell transcriptomic data [109]. There exist a wide range of similar clustering methods, some of which are specifically tailored to the datasets that arise from high-throughput assays. For instance, a range of graph-based approaches, developed to cluster high-throughput data [110,111], have been applied to single-cell expression data in an effort to eliminate some of the effects of outliers and take account of the intrinsic complexity and high levels of noise inherent to these data [112,113].

Machine learning of cellular dynamics

In parallel to the increase in single-cell data, a variety of computational methods have been developed to better understand stem cell differentiation dynamics. Single-cell omics methods are destructive and permit only a single measurement to be taken at one point in time. However, these static snapshots can be used to reconstruct the likely sequence of phenotypic changes that a typical cell will undergo during development, and such trajectory inference techniques are therefore increasingly being used to decipher the dynamics of stem cell commitment [114–117]. Although successful, a major difficulty with trajectory inference is that different trajectories can give rise to the same distribution of cell states and hence inferred trajectories may represent one of many possibilities [118]. In some circumstances, this limitation can be overcome using (even scant) prior knowledge about the interrogated system (such as knowledge of characteristic end points) to derive better approximations of the developmental trajectories, which can then be used to make predictions for further experimental validation, or as the basis for further mechanistic mathematical models [109].

Regulatory network inference

In the absence of prior knowledge on the (causal) relationships between genes and their products (i.e., knowledge of the structure of the underlying gene regulatory network), mathematical models may not appear to be immediately practical. However, knowledge of the causal interactions between features needed to establish a mathematical model can sometimes be learned directly from data using machine learning approaches. There are a number of methods for such "structure learning," the most widely used being Bayesian networks [119]. Bayesian networks have proven useful for the prediction of regulatory interactions in a variety of biomedical contexts [120,121]. Bayesian networks seek to infer a graphical structure—essentially a network wiring diagram, in which each node represents a regulatory species (e.g., a gene or protein) and edges linking two nodes indicate conditional dependence (some of which may be causal), while the absence of edges indicates conditional independence [119]—which is most compatible with the coexpression patterns observed in the data collected. For example, Sachs et al. [121] used a Bayesian network approach to infer a signaling network in human immune cells, in which nodes represent phosphorylated proteins and phospholipids, while edges between nodes indicate conditional dependence, interpreted as a functional regulatory relationship (e.g., activation, inhibition, or coregulation).

The use of Bayesian networks allows for prediction of true functional relationships between genes and proteins of interest. However, due to technical limitations, they are not capable of inferring feedback structures and may contain both true and false negatives and positives. To improve their ability to detect the true underlying dependence structure, interventional data may also be used, in which the expression levels of specific nodes are altered through knockdown or application of small molecule kinase inhibitors to test which interactions are truly functional [121].

Recently, alternative network inference approaches have been developed to overcome some of the limitations intrinsic to the Bayesian approach, such as their inability to detect feedback loops, a network motif commonly observed in stem cell gene regulatory networks [122]. Among these, alternative approaches are methods based on information theory [123], and, increasingly, methods tailored to single-cell transcriptomic data [124]. Notably, aggregate methods that construct consensus regulatory networks from an ensemble of different methods frequently achieve the highest comparable prediction accuracy when benchmarked against known networks [125,126].

In summary, network inference methods can be used to obtain insights from large-scale biological data and the hypotheses generated through network inference can guide further in vitro and in silico experiments to identify the molecular regulatory mechanisms that underpin stem cell differentiation.

From mathematical models to clinical reality

Mathematical methods are routinely used in engineering to optimize complex processes for industrial implementation. Mathematical models can be tremendously powerful yet rely on prior knowledge to enable the formulation of hypotheses in form of a set of equations. In the absence of such prior knowledge, or where data complexity is overwhelming, machine learning can be used to first learn structure in data, categorize observations, and help generate hypotheses for subsequent testing by experiment or formulation of mathematical models. We anticipate that as tissue engineering protocols make their way from

development in the lab to routine use in the clinic, mathematical and machine learning methods will play an increasingly central role in their optimization and scale-up. Mathematical optimization approaches are already being used to design custom scaffolds for tissue regeneration [72,127–129]. For example, Hollister et al. have outlined a strategy for the rational design of porous scaffolds with defined architectural properties that balance conflicting mechanical and transport requirements (reviewed in Ref. [72]). This strategy uses a set of mathematical tools from homogenization theory—which allow macroscopic material and transport properties to be inferred from scaffold microstructure—to aid scaffold design in silico for practical implementation using novel solid free-form fabrication techniques. Such custom approaches have been successfully used to design optimized scaffolds for bone tissue engineering in craniofacial reconstruction, for instance [128]. As another example, Unadkat et al. have recently outlined an algorithm-based strategy to design biomaterial surface micro-topographies that optimally regulate cell function [130]. It is likely that advanced multidisciplinary protocols, such as these, which coordinate mathematical, computational, chemical, and materials engineering techniques, will lead to further advances in scaffold design in the near future. Indeed, design optimization is perhaps the area, in which there is the most potential for genuinely productive application of mathematical and computational principles to tissue engineering and regenerative medicine. Such sophisticated design strategies are likely to become essential to the future of tissue engineering and ultimately will help to take tissue engineering from experimental concept to clinical reality.

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Chapter 3

Moving into the clinic

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Introduction

A high demand for organs needed for transplantation exists in the United States. The staggering number is more than 100,000 on the organ transplant list with approximately 18 people dying daily while waiting for an organ [1]. In contrast, a patient requiring an organ donor is added to the transplant list every 10 minutes [2]. This urgent demand and limited supply of donor organs requires a significant change in the way we address the need for organ transplants. The field of tissue engineering and regenerative medicine has been identified as a promising, innovative path for fulfilling this need. The goal of tissue engineering is to produce constructs that mimic a native organ or structure for replacement or to function as a bridge to encourage the body to regenerate the native structure in vivo. The constructs may include a scaffold component, a cellular component, and biochemical factors/molecules or a combination of these parts (see Fig. 3.1). These therapies have the capacity to replace, repair, or regenerate cells, tissues, and organs to restore normal function in the body. This new era of health care is a paradigm shift from treatment-based to cure-based therapies.

This chapter covers the status of tissue engineering in clinical translation; where we are and where we are going with research and development. It will also provide information on products that are currently in clinical studies as well as those in the market. In this chapter, overview of an academic model with a pathway from proof of concept to final product for implant will be discussed. This translational process includes process and product development (PD), which then transitions to clinical manufacturing along with quality control (QC) testing and quality assurance (QA) oversight in clinical trials. When a project has both an integrated PD approach and a solid design for product manufacturing, a shorter pathway to the clinic may be facilitated. The regulatory considerations for translation, including the relevant FDA regulations and guidance documents that govern tissue-engineered products, and testing in a clinical trial, will also be discussed. Careful review and understanding of the FDA regulations and guidance documents can help accelerate the development process and streamline the pathway to treating patients.

Current state of tissue engineering

Tissue engineering is a term that can be traced back to the 1980s when Fung, a scientist in the field of bioengineering, submitted a proposal to National Science Foundation for engineering a living tissue [3]. This important proposal planted the seed that led to several other organizations and individuals to further develop the field. Drs. Robert Langer and Joseph Vacanti brought clarity and definition to tissue engineering in their article in *Science* (May 1993) where they referenced the

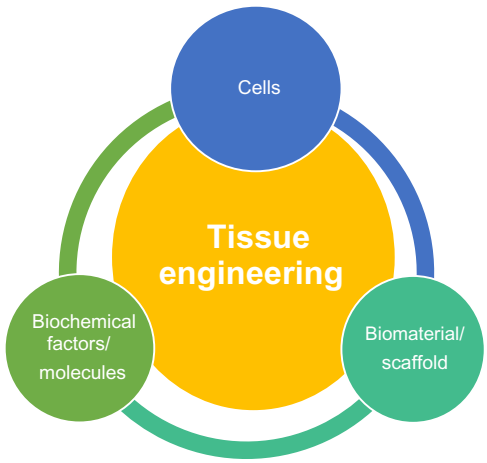

FIGURE 3.1 Components of a tissue engineered construct required to mimic a native organ or structure or to function as a bridge to encourage the body to regenerate the native structure.

Principles of Tissue Engineering. DOI: https://doi.org/10.1016/B978-0-12-818422-6.00003-4

proceedings of the Granlibakken workshop and the discussions on tissue engineering as an interdisciplinary field bringing together engineering and life sciences to produce biologically relevant constructs. Interestingly, in 1994 when searching the term "tissue engineering," only approximately 10 references could be found [3]. From then on, the field grew rapidly. With the field expanding, scientists wondered whether tissue engineering would result in a functional structure that could be implanted in the body. In 2001 another symposium was provided by BECON (National Institutes of Health Bioengineering Consortium), which further provided clarity on tissue engineering, which was described as one area of reparative medicine. Haseltine coined the term "regenerative medicine" to define the in vitro construction of tissues and organs for implant using natural human components and high-technology nonliving materials [4]. In 1999 Dr. Atala, a leader in the field of tissue engineering, discussed the arrangement of tissue-engineered bladder replacement clinical studies in a future perspective article [5]. These were the first published communications of tissue-engineered products moving into the clinic. A later publication suggested the translational pathway of in vitro construction of an organ or tissue on the lab bench to manufacturing a clinically relevant construct for implantation [6]. In 2006 Atala et al. published in Lancet the stunning results of engineered bladders that were implanted in patients requiring cystoplasty [7]. The meetings, publications, and milestones highlighted here represent only some of the work that drove the field to where it is today.

Regenerative medicine as a field has matured into a new era with regulations evolving to ensure safety of these complex products. There has been significant progress in the last few years in moving promising treatments to the clinic where numerous replacement tissues can now be generated de novo. Clinical studies in regenerative medicine include orthobiologics, musculoskeletal, urology, and wound healing. Some of the first examples of commercialized tissue engineering products are dermal substitutes such as Dermagraft (Organogenesis) and Apligraft (Organogenesis) [8,9]. Dermagraft (a skin substitute consisting of cells, an extracellular matrix, and a scaffold) was used for the treatment of diabetic foot ulcers and chronic wounds. Apligraft (a bilayered skin substitute) is approved for both diabetic foot ulcers and venous leg ulcers. After FDA clearance of these products' premarket approval applications, both were released for public use. GINTUIT (Organogenesis and XVIVO, a cellular-based product with bovine collagen) is a sheet applied topically for mucogingival conditions [10]. This product was the first FDA-approved combination product by Center for Biologics Evaluation and Research (CBER) arm of the FDA and the first for use in dental application.

When searching for the term "tissue engineering," the growing field currently has about 113,000 publications in PubMed and close to 84 clinical trials posted on *clinicaltrials.gov*. The Alliance for Regenerative Medicine stated in their 2018 Q3 report that there are 41 clinical trials worldwide in tissue engineering (distinguished from cell or gene therapy) with around a third of those in Phase 3 trials [11]. There are currently a handful of marketed FDA-approved products. The field is starting to mature as more and more products reach market and technologies can accelerate following safety and efficacy demonstration in early clinical studies in patients.

Pathway for clinical translation

One of the most important aspects in the acceleration of clinical translation for regenerative medicine technology is to have a comprehensive understanding of the regulations that are required to move a product to market. Without knowledge of the regulatory requirements, one could spend excessive time and resources on the path to clinic. The clinical translational pathway starts with the end in mind: the patient, the condition to be treated, and the form and function of the final clinical product. So first and foremost, it is critical to have the clinician involved as the treatment and study model is being conceptualized. Clinicians have a deep understanding of what is required at the bedside; what current treatments are lacking and how the proposed product can be more efficient; what the cost considerations are; and what approach will provide a better quality of life for their patients. The clinicians also have the best understanding in terms of patient access and urgency for treatment. For example, if a physician is treating a stroke patient post episode and the treatment is most effective when administered immediately, culturing autologous cells from the patient for weeks would not be a viable option; however, having an off-the-shelf allogeneic cellular product could be a possibility.

The general translational pathway is illustrated in Fig. 3.2. Transfer of technology from the lab bench to PD and then on to manufacturing requires an enormous amount of time and efforts to move products through the translation pathway, especially if these units are located at different sites. Methods often have to be observed and performed multiple times during technology transfer for all aspects to translate successfully. For example, if tissue-derived biomaterial is used to produce an engineered muscle product, harvesting a specific region of tissue from an organ is required before decellularization (cell removal). In order to harvest the tissue, it is important to understand the anatomy for identifying the tissue layers, proper orientation of the tissue as well as methods to dissect and separate the layers to obtain target segments. It is also crucial to understand what the ideal target

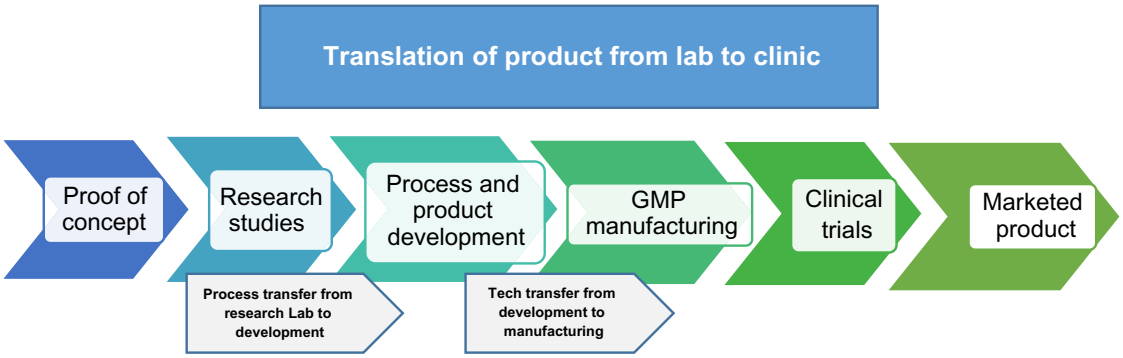

FIGURE 3.2 General pathway for clinical translation.

tissue should look like after separation, the amount of force required to separate the layers without damaging the tissue, and what a normal biomaterial source organ should look like and whether organs should be discarded due to disease or damage. The technology transfer of just this one step may take several training sessions with multiple batches of incoming organs in order to encounter different scenarios that require troubleshooting. Since this is the first step to manufacturing the biomaterial, all subsequent steps in the process could not be worked on until this step is ironed out. Therefore having all teams at a single site to answer questions and address any issues that emerge can accelerate the translation process.

One important academic model developed for clinical translation is an all-inclusive model where the research, preclinical studies, PD, manufacturing, quality testing, and QA oversight for the technology are retained in-house, and projects move from proof of concept all the way to early phase clinical trials in one location. Benefits of this model include a multidisciplinary team that can meet regularly and tackle issues quickly as they arise. The site is equipped with the academic power to develop innovative technology by established researchers funded to solve specific issues in health care. The researcher can perform the in vitro studies and preclinical studies providing pilot data that support the technology. Definitive preclinical research studies can be performed under FDA Good Laboratory Practices (GLP) to test for efficacy, toxicity, tumorigenicity, and biodistribution. A research core facility provides equipment and expertise to facilitate data collection and translation. The in-house translational core or clinical translational unit includes PD, manufacturing, QC, QA, and a regulatory affairs team. The regulatory team prepares briefing documents and communicates with regulators. These embedded services can provide acceleration of PD and intellectual property (IP). An in-house legal team provides guidance on IP and licensing to investigators. Having all stages of the translational pathway under one umbrella can save time and resources, allowing for development of multiple clinical products simultaneously to be incorporated early on and throughout the process.

When definitive preclinical research studies are performed under the all-inclusive academic model, they can be performed with the knowledge of the data that will be used to support an FDA IND (Investigational New Drug) submission, which means that studies will be conducted under GLP documentation standards. Often, if research is conducted in an academic setting, the investigator and any collaborators plan the animal studies with only the goal of publication in mind. When the project shows promise for translation after the study, the results can be included in FDA IND submissions. Under the all-inclusive academic model, the study model and experiment plan can be discussed with the FDA during pre-IND (PIND) discussions to confirm the animal model, and the study fully supports the translation of the product to the clinic.

A clinical translational unit in an academic environment can benefit moving technology to early phase clinical trials by submitting IND filings, transferring technology, producing clinical products, testing clinical products, and coordinating and training clinicians with product responsibilities. The scope of each project will be dependent on the phase of translation the project is in when introduced to the translational unit, thereby dictating the amount of involvement required by the team. The clinical translational unit has the goal of facilitating translation of scientific discoveries into clinical therapies, while meeting the requirements of the US FDA to ensure safety and compliance. The clinical translational unit develops and manufactures products and provides regulatory support to navigate the pathway to early phase clinical trials. An overview of this process is presented in Fig. 3.3.

Following proof of concept and benchtop research activities, PD occurs with the aim of preparing the product for human clinical use. During this stage the laboratory investigator will transfer the technology to the PD team. The PD team then develops and refines the test

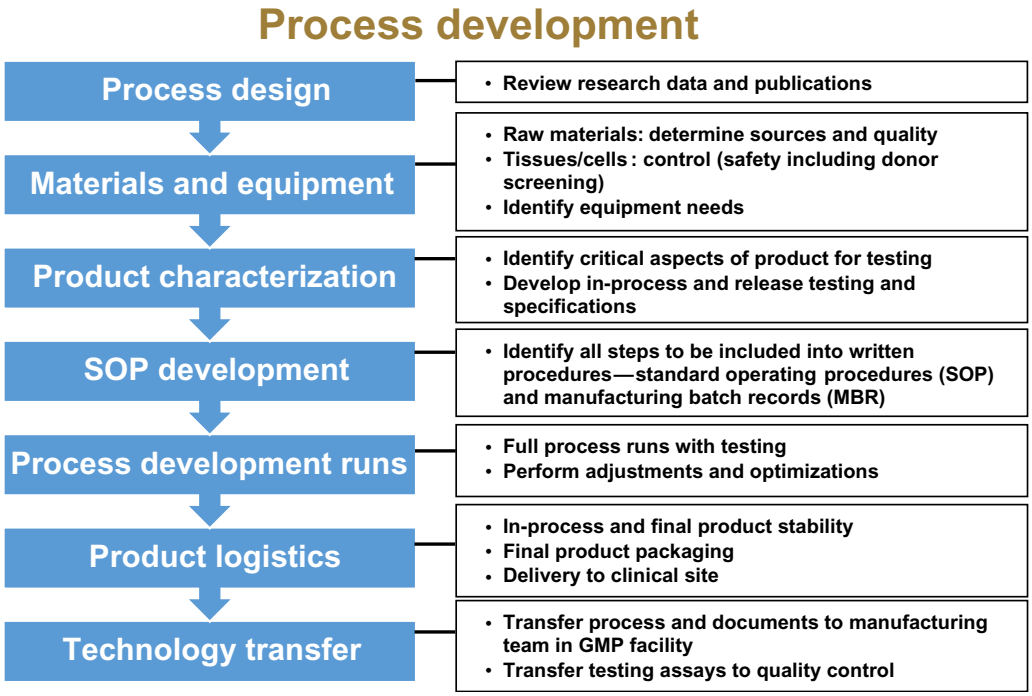

FIGURE 3.3 Components of the process development phase of clinical translation.

article for definitive preclinical GLP studies. Along with preclinical management staff, they assist with coordination and/or execution of GLP studies that meet FDA requirements. PD develops processes and sources materials suitable for human clinical trials, ensuring compliance with regulations. All materials in direct contact with the manufactured product need to be biocompatible and bioreactors, if applicable, should be constructed using USP Class VI materials. The transition of the technology from treating an animal to human requires most of the PD focus. When large animal models such as pigs or sheep are used, often the size of the construct and the number of cells required for the product are not significantly different than the human construct. However, if animal models used smaller constructs, then there can be significant effort in developing new bioreactors to support construct maturation, and new cell culturing techniques and media optimization to support expansion of cells to larger numbers. PD develops tests and assays to characterize the product going into manufacturing. To ensure the product is made consistently, the assays should have precise specification criteria. The testing should be performed in-process and at the time of final product. A large number of technical studies are performed during the development process. For instance, certain reagents or instruments used to manufacture the product during the development phase may not be suitable for a clinical grade product. If possible, appropriate clinical grade reagents and surgical grade instruments should be selected. The technical study would then compare producing the product with the traditional reagents and instruments and the newly identified materials. Other technical studies may focus on shifting the process to closed systems to reduce contamination risks or handling risks, which may harm the operator or the product. PD also gathers data required to support the *I*N*i*tial *T*argeted *E*ngagement for *R*egulatory *A*dvice on *C*BER Produc*T*s (INTERACT), or a PIND application, to the FDA. They perform PD process runs with testing to optimize and improve on the workflow. PD will identify product stability at all hold points throughout the process. Product packaging and logistics related to final transport to the clinical site is also developed. Once a process is refined and can be performed following written standard operating procedures (SOPs), the PD team works with manufacturing to transition development processes into formal SOPs and manufacturing batch records (MBRs). The PD team works closely with the manufacturing and QC teams to transition the projects into the good manufacturing practices/good tissue practices (GMP/GTPs) facility. An overview of this late translation process is shown in Fig. 3.4.

The role of manufacturing is to perform activities to produce cell and tissue products for clinical trials in accordance with GMP/GTP requirements or to manufacture FDA-registered products in accordance with GTP. The manufacturing team works with the PD team to transition processes into SOPs and MBRs for the GMP/GTP environment. They perform manufacturing engineering

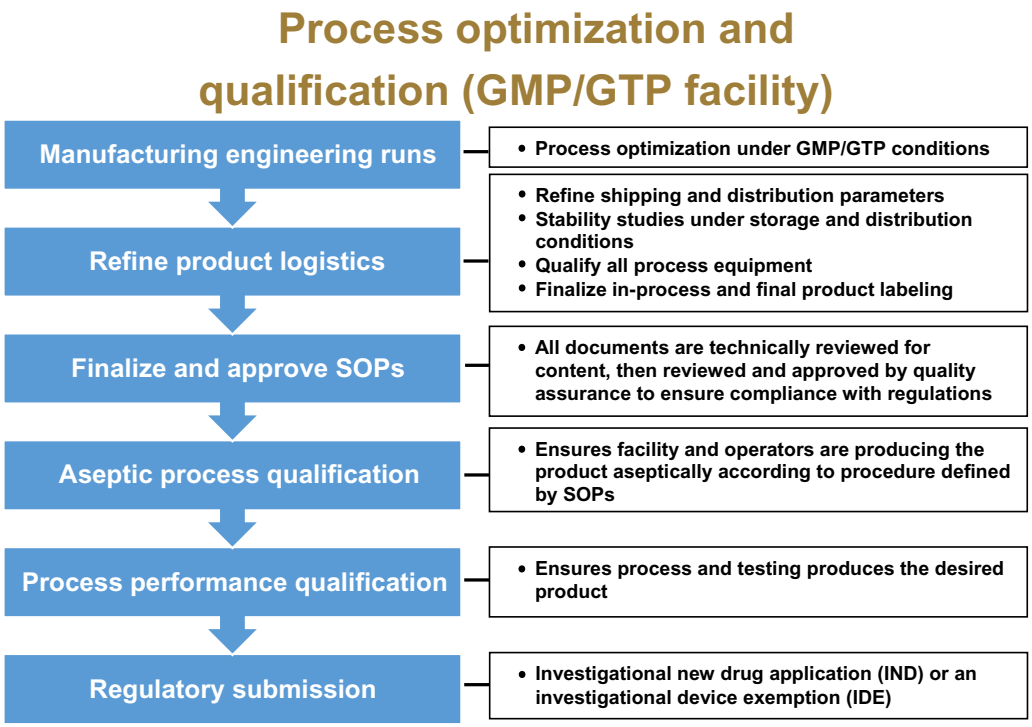

FIGURE 3.4 Components of late translation: process optimization and qualification for manufacturing and regulatory filing.

runs within the GMP/GTP facility and optimize the process if necessary. The product logistics are further refined and all processing equipment must be qualified for installation and operation (IOQ). Preventative maintenance and calibration services must be performed on equipment regularly to ensure proper function. SOP and MBR documents are finalized with technical review, then reviewed and approved by QA. All manufacturing personnel must be trained to manufacture the desired product safely, effectively, and efficiently. The training should be clearly documented, and no operator will perform processing without documented training. Clinical processing includes a primary operator and secondary operator that assists and verifies that each critical step is performed properly. For certain processes, multiple primary operators may be required. To ensure sufficient materials inventory, control, and documentation in advance of each process, a materials kit is made with separate bins for different storage temperatures, such as room temperature, refrigerator, −20°C or −80°C freezer. Tracking numbers and expiration dates of all materials in the kits are recorded to ensure traceability. Before clinical processing can occur, two important qualifications to be completed are the aseptic process qualification (APQ) and the process performance qualification (PPQ). The APQ, or process simulation, uses a growth promoting media in place of all reagents and ensures the GMP/GTP facility maintains environmental control during processing to produce an aseptic product while performing the procedure per SOPs. The APQ may not always be necessary if the product is terminally sterilized prior to administration to a patient, but the production area must be otherwise evaluated and monitored to ensure it is appropriately free of particulates and adventitious agents during production. The PPQ ensures that the finalized SOPs, MBRs, environment, and trained personnel can produce the desired product and pass all in-process and final release testing. The PPQ run (s) are also carried out as if producing the final clinical product; by doing so, any issues to the process can be identified and resolved prior to manufacturing the product for first in human application. An IND application or an investigational device exemption can be submitted at this point. The submissions are built on data from PD, QC testing, and manufacturing, which includes all technical studies and engineering runs. One notable caveat is that the PPQ may be performed (or may need to be repeated) after the FDA approves the application; thus, any changes required by the FDA are be included in the final PPQ.

Once approval is granted from the regulatory agencies, manufacturing can begin, and patients can be recruited for the study. The manufacturing process for early phase clinical trials or registered products may include tissue receipt, cell isolation and expansion, scaffold preparation, cell seeding onto scaffold, packaging, and shipment to the clinical site. Samples are collected throughout the process and submitted to QC for in-process testing to characterize

the clinical product at various stages of production and then ultimately final release testing prior to delivery to the patient. During clinical manufacturing and QC testing, all activities require documentation to ensure concurrent recording of processes/testing and traceability of all in-process chemicals, materials, and equipment. All written records must follow good documentation practices. Manufacturing and QC testing is monitored by QA to ensure procedures are followed to ensure patient safety.

QA establishes policies and procedures and QC performs testing to ensure compliance with regulatory and internal requirements. QA has responsibility of ensuring documentation is reviewed and meets regulatory requirements after manufacturing has produced the product. QA is also responsible for a document management system and ensuring appropriate record archiving takes place. They also govern and control incoming materials, reagents, and consumables to ensure only released items meeting specifications are used in the GMP/GTP facility for the manufacturing of a clinical product. They are responsible for facility oversight, ensuring that PD and manufacturing activities take place in a controlled environment with equipment that is calibrated and validated to meet GMP and GTP requirements. QA is responsible for the master validation plan of the facility and will perform regular in-house audits of the process and facility. QA certifies the final product at time of release to clinical site by reviewing all documentation and testing results to ensure all procedures were properly followed and the product has met all specifications. QA maintains all relevant documents that support the GMP/GTP facility or any clinical product in order to be ready for FDA inspections that can occur at any time.

A critical piece of translation is regulatory affairs, which manages and directs strategic and operational aspects of regulatory matters for technologies in regenerative medicine including cell and gene therapy, tissue engineering, biomaterials, and devices. This team maintains regulatory and compliance processes. It prepares, reviews, and evaluates documents for submission to regulatory authorities. The required expertise of this team is to provide focused regulatory drivers for accelerated pathways to control time and cost of translation.

Project management (PM) is an important link to facilitate organization, communication and information processing within the translational unit for both preclinical and clinical effort. PM coordinates individual team member's efforts and tracks outstanding items to prevent project delays. They promote efficient purchasing of reagents, materials, supplies equipment, and service agreements. Administrative tasks for the project are also performed by the PM.

The overall head or director of the translation unit should have extensive experience in preclinical, PD, manufacturing, QC, QA, and regulatory affairs, but with a strong emphasis on the production of a clinical product. The head of translation oversees strategic and operational aspects of global regulatory matters for all products moving to commercialization under FDA jurisdiction and research and development projects, including the regulatory and compliance processes, team management, milestone achievement, as well as the preparation, review and evaluation of documents for submission to regulatory authorities. Responsibilities for the head of translation includes setting priorities, providing strategic input, identifying and assessing regulatory risks, understanding global imperatives including market-related drivers, interfacing with regulatory authorities, keeping up with US and global regulatory requirements and guidance, and providing oversight and review of key regulatory documents. Under the direction of the head of translation, the teams work alongside researchers and clinicians to facilitate the translational process in a safe and timely manner that supports an overall mission of facilitating translation of the research from the bench to the bedside.

Regulatory considerations for tissue engineering

Regulatory requirements for biologics used in tissue engineering follows GMP and GTP regulations. GMP were initially developed for pharmaceuticals or drug production, which are easier processes and straight forward when dealing with defined products such as small molecule drugs as opposed to viable organisms or cells. Oversight of therapeutics derived from biological sources under the US FDA has provided clarity in regulations through guidance documentation and communication (written and presentations) to facilitate understanding of requirements to move a technology to the market. The FDA provides more guidance for investigators now than at any prior point in its history. This strengthened relationship with regulators provides an opportunity to transfer the science to the bedside in an efficient, safe, and cost-conscious manner.

Growth in the field of regenerative medicine and tissue engineering has led to an elevated use of novel therapies to repair or replace tissues and organs and further requirements to provide safety in clinical products. Global regulatory standards were established with the goal of reducing the risk of communicable transmissible disease. As regulators are caretakers of the health-care structure, they need to be able to balance risks and benefits when assessing technology that may have lifesaving capabilities. In 2011 the US FDA issued a final rule requiring human cell, tissue, and cellular and tissue-based product (HCT/P) Establishments to be compliant with current

GTPs covering collection, donor screening, donor testing, processing, labeling, packaging, storage, and distribution of HCT/Ps [12]. The regulations for GTPs are found in 21 CFR 1271 Subpart D [13] and cover facilities, environmental controls, equipment, supplies and reagents, recovery, processing and processing controls, labeling controls, storage, and distribution of products. The regulation was drafted for products under the CBER. The GTP regulation covers human cells, tissues, and tissue-based products in addition to any cell product procured from a donor and implanted or transplanted into a recipient.

FDA provides two tiers of regulation for HCT/Ps according to a risk-based approach. HCT/Ps under 21 CFR 1271 [13,14] and Section 361 of the Public Health Service Act (PHSA) are regulated solely as 361 products when they meet criteria listed in 21 CFR 1271.10(a) [13]:

- minimally manipulated;
- intended for a homologous use only as reflected by the labeling, advertising, or other indications of the manufacturer's objective intent; and
- not combined with another article.

HCT/Ps not covered by Section 361 of the PHSA are covered under Section 351 of the PHSA (351), which is also regulated by CBER. This tier includes products that do not meet all the criteria in 21 CFR 1271.10(a) and are regulated as drugs and/or biological products. These products under Section 351 of the PHSA require premarket approval by FDA. Minimal manipulation and homologous use are further defined in a guidance document published by FDA in 2017 [14], which provides a resource for clarity on regulations required for the HCT/Ps. In summary, HCT/Ps regulated as a 361 do not require premarket approval and are covered under 21 CFR 1271 for GTPs. HCT/Ps regulated as a 351 product requires premarket approval and are covered under both 21 CFR 210 and 211 for GMP and 21 CFR 1271 for GTP [13]. The 351 products are regulated as an IND, while 361 products do not require an IND since they are geared toward the prevention of communicable diseases and infection, but they still include donor eligibility requirements. Tissue-engineered constructs are generally combination products that include cells and a scaffold and are regulated as a 351, thereby requiring an IND. The scaffold is considered a device and regulated by the Center for Devices and Radiological Health and device regulations under the general review of CBER according to the primary mode of action of the product.

A tissue-engineered construct requires an IND and compliance with GMP and GTP. GTP controls all steps in recovery, donor screening, donor testing, processing, storage, labeling, packing, and distribution according to 21 CFR 1271.150(a). Core requirements as referenced in 21 CFR 1271.150(b) include

- facilities;
- environmental controls;
- equipment;
- supplies and reagents;
- recovery;
- processing and process controls;
- labeling controls;
- storage;
- receipt, predistribution shipment, and distribution of an HCT; and
- donor eligibility determinations, donor screening, and donor testing.

For a 351 HCT/P, GMP requirements cover areas such as documentation, validation, facilities, equipment, personnel, training, controlled documentation, manufacturing, process control, labeling, packaging, storage, and distribution. These are covered under 21 CFR Part 210

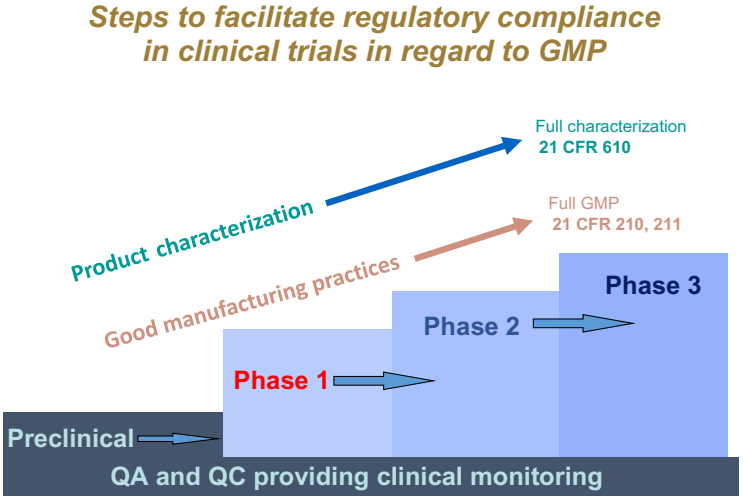

FIGURE 3.5 Steps to facilitate regulatory compliance in clinical trials for tissue engineered products.

GMP for manufacturing, processing, packaging, and storage of drug products and 21 CFR Part 211 GMP for finished pharmaceutical products (Fig. 3.5).

In September 2004 the US Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, CBER, and Office of Regulatory Affairs released a Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice to assist manufacturers in complying with the GMP regulations for pharmaceutical manufacture [12]. Then, in July 2008, the FDA issued a specific guidance for industry: current good manufacturing practice (CGMP) for Phase 1 Investigational Drugs to replace the original Phase 1 CGMP guidance issued in 1991 (20 and 21). The goal of this 2008 guidance is to further clarify the requirements for CGMP compliance, set forth more broadly in the 2004 and 1991 guidance documents, but for the manufacture of investigational new drugs used in Phase 1 clinical trials. It explains these drugs are exempt from complying with 21 CFR part 211 under 21 CFR 210.2(c) (Phase 1 investigational drugs); the original guidance was directed mostly at large, repetitive, commercial batch manufacturing of products. The 1991 guidance is still relevant for Phase 2 and 3 products, which means that once products reach this stage, they will be required to fully comply with 21 CFR 210 and 211. By Phase 3, products will also comply with 21 CFR 610, which outlines testing criteria to fully characterize the product. For example, potency tests should be fully developed prior to commercialization [15]. The progression of increasing compliance and regulatory oversight is graphically presented in Fig. 3.5. As the tissue engineering field is still considered up and coming, regulatory bodies are adapting traditional regulations and creating new regulations to accommodate the field. After feedback from translational teams, additional adjustments may be made by regulatory agencies; hence, throughout translation, regulatory considerations should be monitored closely to ensure compliance.

Conclusion

With an aging population in the United States and globally, and with demand for organ transplants exceeding availability, regenerative medicine becomes a critical goal of health care for sustainability as it will provide a significant improvement in quality of health care for the future. One important aspect of regenerative medicine is tissue engineering where the cellular material along with extracellular components and a scaffold are used in the construction of an organ or tissue. In this chapter the translational process was discussed from proof of concept to clinical trials. To reduce the total time for the translation process, regulatory considerations should be monitored throughout the process to efficiently translate treatments to patients to improve their quality of life.

Acknowledgment

We acknowledge Sherrie Mechum for the formatting of the figures used in this chapter.

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Further reading

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products (HCT/Ps). Guidance for Industry. Available from: <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/current-good-tissue-practice-cgtp-and-additional-requirements-manufacturers-human-cells-tissues-and>; 2011 [cited May 6, 2019].

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Part One

The basis of growth and differentiation

Chapter 4

Molecular biology of the cell

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To a naive observer the term "tissue engineering" might seem a contradiction in terms. The word "engineering" tends to conjure up a vision of making objects from hard components, such as metals, plastics, concrete, or silicon, which are mechanically robust and will withstand a range of environmental conditions. The components themselves are often relatively simple, and the complexity of a system emerges from the number and connectivity of the parts. By contrast, the cells of living organisms are themselves highly delicate and highly complex. Despite our knowledge of a vast amount of molecular biology detail concerning cell structure and function, their properties are still understood only in qualitative terms, and so any application using cells requires a lot of craft skill as well as rational design. What follows is a very brief account of cell properties, intended for newcomers to tissue engineering who have an engineering or physical science background. It is intended to alert the readers about some of the issues involved in working with cells and to pave the way for an understanding of how cells form tissues and organs, topics dealt with in more detail in the later chapters. As it comprises very general material, it is not specifically referenced, although some further reading is provided at the end.

Cells are the basic building blocks of living organisms in the sense that they can survive in isolation. Some organisms, such as bacteria, protozoa or many algae, actually consist of single free-living cells. But most cells are constituents of multicellular organisms, and although they can survive in isolation, they need very carefully controlled conditions in order to do so. A typical animal cell suspended in liquid is a sphere of the order of about 20 μm diameter (Fig. 4.1). Most cells will not grow well in suspension, and so they are usually grown attached to a substrate, where they flatten and may be quite large in horizontal dimensions but only a few microns in vertical dimension. All eukaryotic cells contain a nucleus in which is located the genetic material that ultimately controls everything that the cell is composed of and all the activities it carries out. This is surrounded by cytoplasm, which has a very complex structure and contains substructures called organelles which are devoted to specific biochemical functions. The outer surface of the cell is the plasma membrane, which is of crucial importance as it forms the frontier across which all materials must pass on their way in or out. The complexity of a single cell is awesome since it contains thousands of different types of protein molecules, arranged in many very complex multimolecular aggregates comprising both hydrophobic and aqueous phases and also many thousands of low molecular weight metabolites, including sugars, amino acids, nucleotides, fatty acids, and phospholipids. Although some individual steps of metabolism may be near to thermodynamic equilibrium, the cell as a whole is very far from equilibrium and is maintained in this condition by a continuous interchange of substances with the environment. Nutrients are chemically transformed with release of energy which is used to maintain the structure of the cell and to synthesize the tens of thousands of different macromolecules on which its continued existence depends. Maintaining cells in a healthy state means providing them with all the substances they need, in the right overall environment of temperature, solute concentration, and substratum, and also continuously removing all potentially toxic waste products which the cells continuously produce.

The cell nucleus

The nucleus contains the genes that control the life of the cell. A gene is a sequence of DNA that codes for a protein, or for a nontranslated RNA, and it is usually considered also to include the associated regulatory sequences as well as the coding region itself. The vast majority of eukaryotic genes are located in the nuclear chromosomes, although there are also a few genes carried in the DNA of

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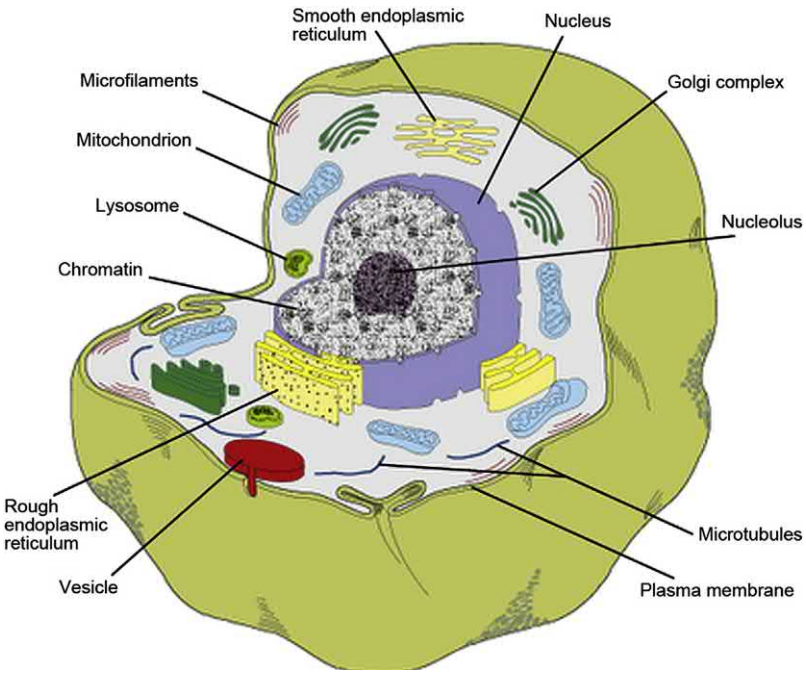

FIGURE 4.1 Structure of a generalized animal cell. (http://scienceblogs.com/clock/2006/11/09/cell-structure/ accessed 15.05.13).

mitochondria and chloroplasts. The genes encoding nontranslated RNAs include those for ribosomal or transfer RNAs and also for a large number of microRNAs and long noncoding RNAs that can modulate the expression of protein coding genes. The total number of protein coding genes in vertebrate animals is about 25,000. The idea that every cell nucleus carries an identical set of genes derived from the parents is generally true, but there are some irreversible DNA modifications. The B and T lymphocytes of the immune system undergo irreversible rearrangements in the genes encoding antibodies and T-cell receptors, respectively. There are many transposable elements within the genome that may move to other locations during development. In addition all cells, both in vivo and in cell culture, continuously accumulate somatic mutations.

The nuclear DNA is complexed into a higher order structure called chromatin by the binding of basic proteins called histones. Protein coding genes are transcribed into messenger RNA (mRNA) by the enzyme RNA polymerase II. Transcription commences at a transcription start sequence and finishes at a transcription termination sequence. Genes are usually divided into several exons, each of which codes for a part of the mature mRNA. The primary RNA transcript is extensively processed before it moves from the nucleus to the cytoplasm. It acquires a "cap" of methyl guanosine at the 5′ end, and a polyA tail at the 3′ end, both of which stabilize the message by protecting it from attack by exonucleases. The DNA sequences in between the exons are called introns and the portions of the initial transcript complementary to the introns are removed by splicing reactions catalyzed by "snRNPs" (small nuclear ribonucleoprotein particles). It is possible for the same gene to produce several different mRNAs as a result of alternative splicing, whereby different combinations of exons are spliced together from the primary transcript. In the cytoplasm, the mature mRNA is translated into a polypeptide by the ribosomes. The mRNA still contains a 5′ leader sequence and a 3′ untranslated sequence, flanking the protein coding region, and these untranslated regions may contain specific sequences responsible for translational control or intracellular localization.

Control of gene expression

There are many genes whose products are required in all tissues at all times, for example, those concerned with basic cell structure, protein synthesis, or metabolism. These are referred to as "housekeeping" genes. But there are many others whose products are specific to particular cell types and indeed the various cell types differ from each other, because they contain different repertoires of proteins. This means that the control of gene expression is central to tissue engineering. Control may be exerted at several points. Most common is control of transcription, and we often speak of genes being "on" or "off" in particular situations, meaning that they are or are not being transcribed. There are also many examples of translational regulation, where the mRNA exists in the cytoplasm but

is not translated into protein until some condition is satisfied. Control may also be exerted at the stage of nuclear RNA processing or indirectly via the stability of individual mRNAs or proteins.

Control of transcription depends on regulatory sequences within the DNA and on proteins called transcription factors that interact with these sequences. The promoter region of a gene is the region to which the RNA polymerase binds, just upstream from the transcription start site. The RNA polymerase is accompanied by a set of general transcription factors, which together make up a transcription complex. In addition to the general factors required for the assembly of the complex, there are numerous specific transcription factors that bind to specific regulatory sequences that may be either adjacent to or at some distance from the promoter (Fig. 4.2). These are responsible for controlling the differences in gene expression between different types of cell.

Transcription factors

Transcription factors are the proteins that regulate transcription. They usually contain a DNA-binding domain and a regulatory domain, which will either upregulate or repress transcription. Looping of the DNA may bring these regulatory domains into contact with the transcription complex with resulting promotion or inhibition of transcription. There are many families of transcription factors, classified by the type of DNA-binding domain they contain, such as the homeodomain or zinc-finger domain. Most are nuclear proteins, although some exist in the cytoplasm until they are activated and then enter the nucleus. Activation often occurs in response to intercellular signaling (see later). One type of transcription factor, the nuclear receptor family, is directly activated by lipid-soluble signaling molecules such as retinoic acid or glucocorticoids, which can penetrate cell membranes easily.

Each type of DNA-binding domain in a protein has a corresponding type of target sequence in the DNA, usually 20 nucleotides or less. The activation domains of transcription factors often contain many acidic amino acids making up an "acid blob," which accelerates the formation of the general transcription complex. Transcription factors often recruit histone acetylases which open up the chromatin by neutralizing amino groups on the histones by acetylation and allow access of other proteins to the DNA. Although it is normal to classify transcription factors as activators or repressors of transcription, their action is also sensitive to context, and the presence of other factors may on occasion cause an activator to function as a repressor, or vice versa.

Other controls of gene activity

The chromosomal DNA is complexed with histones into nucleosomes and is coiled into a 30 nm diameter filament, which is in turn arranged into higher order structures. This complex of DNA and proteins is referred to as chromatin, and the structure of chromatin is very important for gene regulation. In much of the genome the nucleosomes are to some extent mobile, allowing access of transcription factors to the DNA. This type of chromatin is called euchromatin. In other regions the chromatin is highly condensed and inactive, then being called

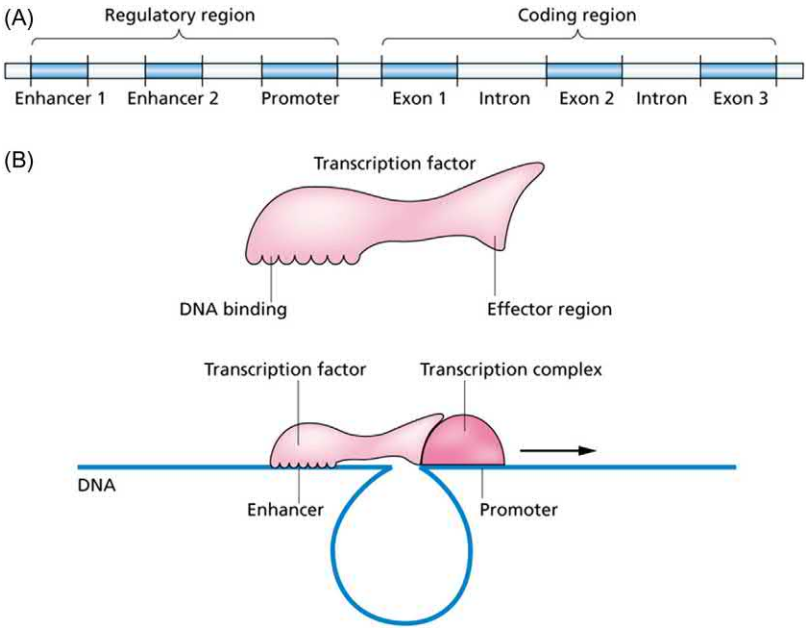

FIGURE 4.2 (A) Structure of a typical gene. (B) Operation of a transcription factor. *From Slack JMW. Essential developmental biology. 3rd ed. Oxford: Wiley-Blackwell; 2012 [1].*

heterochromatin. In the extreme case of the nucleated red blood cells of nonmammalian vertebrates, the entire genome is heterochromatic and inactive. Chromatin structure is regulated to some degree by protein complexes (such as the polycomb and trithorax groups), which affects the expression of many genes but are not themselves transcription factors. An important element of the chromatin remodeling is the control through acetylation of lysines on the exposed N-termini of histones. This partially neutralizes the binding of the histones to the negatively charged phosphodiester chains of DNA, so opens up the chromatin structure and enables transcription complexes to assemble on the DNA. The degree of histone acetylation is controlled, at least partly, by DNA methylation, because histone deacetylases are recruited to methylated regions and will tend to inhibit gene activity in these regions.

DNA methylation occurs on cytosine residues in CG sequences of DNA. Because CG on one strand will pair with GC on the other, antiparallel, strand, potential methylation sites always lie opposite one another on the two strands. There are several DNA methyl transferase enzymes, including de novo methylases that methylate previously unmethylated CGs, and maintenance methylases that methylate the other CG of sites bearing a methyl group on only one strand. Once a site is methylated, it will be preserved through subsequent rounds of DNA replication, because the hemimethylated site resulting from replication will be a substrate for the maintenance methylase. There are many other chemical modifications of the histones in addition to acetylation, including methylation of sites within the histone polypeptides, and these modifications can also be retained on chromosomes when the DNA is replicated. So both DNA methylation and histone modifications provide means for maintaining the state of activity of genes in differentiated cells even after the original signals for activation or repression have disappeared.

The cytoplasm

The cytoplasm consists partly of proteins in free solution, although it also possesses a good deal of structure that can be visualized as the cytoskeleton (see later). Often considered to be in free solution, although more likely in macromolecular aggregates, are the enzymes that carry out the central metabolic pathways. In particular the pathway called glycolysis leads to the degradation of glucose to pyruvate. Glucose is an important metabolic fuel for most cells. Mammalian blood glucose is tightly regulated at around 5−6 mM, and glucose is a component of most tissue culture media. Glycolysis leads to the production of two molecules of adenosine triphosphate (ATP) per molecule of glucose, with a further 36 molecules of ATP produced by oxidative phosphorylation of the pyruvate. This ATP is needed for a very wide variety of synthetic and biochemical maintenance activities. Apart from the central metabolic pathways, the cell is also engaged in the continuous synthesis and degradation of a wide variety of lipids, amino acids, and nucleotides.

The cytoplasm contains many types of organelle that are structures composed of phospholipid bilayers. Phospholipids are molecules with a polar head group and a hydrophobic tail. They tend to aggregate to form structures in which all the head groups are exposed on the surface and the hydrophobic tails associate with each other in the center. Most cell organelles are composed of membranes comprising two sheets of phospholipid molecules with their hydrophobic faces joined. The mitochondria are the organelles responsible for oxidative metabolism, as well as other metabolic processes such as the synthesis of urea. They are composed of an outer and an inner phospholipid bilayer. The oxidative degradation of sugars, amino acids, and fatty acids is accompanied by the production of ATP. Pyruvate produced by glycolysis is converted to acetyl CoA, also formed by the degradation of fatty acids and amino acids, and this is oxidized to two molecules of CO_2 by the citric acid cycle, with associated production of ATP in the electron transport chain of the mitochondria. Because of the importance of oxidative metabolism for ATP generation, cells need oxygen to support themselves. Tissue culture cells are usually grown in atmospheric oxygen concentration (about 20% by volume), although the optimum concentration may be somewhat lower than this since the oxygen level within an animal body is normally lower than the external atmosphere. Too much oxygen can be deleterious, because it leads to the formation of free radicals that cause damage to cells. Tissue culture systems are therefore often run at lower oxygen levels, such as 5%. The oxidation of pyruvate and acetyl CoA also results in the continuous production of CO_2 which needs to be removed to avoid acidification of the culture medium.

Among the organelles in the cytoplasm is the endoplasmic reticulum, which is a ramifying system of phospholipid membranes. The interior of the endoplasmic reticulum can communicate with the exterior medium through the exchange of membrane vesicles with the plasma membrane. Proteins that are secreted from cells, or that come to lie within the plasma membrane, are synthesized by ribosomes that lie on the cytoplasmic surface of the endoplasmic reticulum, and the products are passed through pores into the endoplasmic reticulum lumen. From here they move to the Golgi apparatus, which is another collection of internal membranes, in which carbohydrate chains are added. From there they move to the cell surface or the exterior medium. Secretion of materials is a very important function of all cells, and it needs to be

remembered that their environment in tissue culture depends not only on the composition of the medium that is provided but also on what the cells themselves have been making and secreting.

Intracellular proteins are synthesized by ribosomes in the soluble cytoplasm. There is a continuous production of new protein molecules, the composition depending on the repertoire of gene expression of the cell. There is also a continuous degradation of old protein molecules, mostly in a specialized structure called the proteasome. This continuous turnover of protein requires a lot of ATP.

The cytoskeleton

The cytoskeleton is important for three distinct reasons. First, the orientation of cell division may be important. Second, animal cells move around a lot, either as individuals or as part of moving cell sheets. Third, the shape of cells is an essential part of their ability to carry out their functions. All of these activities are functions of the cytoskeleton. The main components of the cytoskeleton are microfilaments, microtubules, and intermediate filaments. Microtubules and microfilaments are universal constituents of eukaryotic cells, while intermediate filaments are found only in animals. Microfilaments are made of actin, microtubule of tubulin, and intermediate filaments are composed of different proteins in different cell types: cytokeratins in epithelial cells, vimentin in mesenchymal cells, neurofilament proteins in neurons, and glial fibrilliary acidic protein in glial cells.

Microtubules

Microtubules (Fig. 4.3) are hollow tubes of 25 nm diameter composed of tubulin. Tubulin is a generic name for a family of globular proteins that exist in solution as heterodimers of α- and β-type subunits and is one of the more abundant cytoplasmic proteins. The microtubules are polarized structures with a minus end anchored to the centrosome and a free plus end at which tubulin monomers are added or removed. Microtubules are not contractile but exert their effects through length changes based on polymerization and depolymerization. They are very dynamic, either growing by addition of tubulin monomers, or retracting by loss of monomers, and individual tubules can grow and shrink over a few minutes. The monomers contain guanosine triphosphate (GTP) bound to the b subunit, and in a growing plus end, this stabilizes the tubule. But if the rate of growth slows down, hydrolysis of GTP to guanosine diphosphate (GDP) will catch up with the addition of monomers. The conversion of bound GTP to GDP renders the plus end of the tubule unstable, and it will then start to depolymerize. The drugs colchicine and colcemid bind to monomeric tubulin and prevent polymerization and among other effects this causes the disassembly of the mitotic spindle. Because these drugs cause cells to become arrested in mitosis, they are often used in studies of cell division kinetics.

The shape and polarity of cells can be controlled by locating capping proteins in particular parts of the cell cortex, which bind the free plus ends of the microtubules and stabilize them. The positioning of structures within the cell also depends largely on microtubules. There exist special motor proteins that can move along the tubules, powered by hydrolysis of ATP, and thereby transport other molecules to particular locations within the cell. The kinesins move toward the plus ends of the tubules while the dyneins move toward the minus ends. Microtubules are prominent during cell division. The minus ends of the tubules originate in the centrosome, which is a microtubule-organizing center able to initiate the assembly of new tubules. In mitotic prophase the centrosome divides, and each of the radiating sets of microtubules becomes known as an aster. The two asters move to the opposite sides of the nucleus to become the two poles of the mitotic spindle. The spindle contains two types of microtubules. The polar microtubules meet each other near the center and become linked by plus-directed motor proteins. These tend to drive the poles apart. Each chromosome has a special site called a kinetochore that binds another group of microtubules called kinetochore microtubules. At anaphase the kinetochores of homologous chromosomes separate. The polar microtubules continue to elongate while the kinetochore microtubules shorten by loss of tubulin from both ends and draw the chromosome sets into the opposite poles of the spindle.

Microfilaments

Microfilaments (Fig. 4.4) are polymers of actin, which is the most abundant protein in most animal cells. In vertebrates, there are several different gene products of which α actin is found in muscle and β/γ actins in the cytoskeleton of nonmuscle cells. For all actin types the monomeric soluble form is called G-actin. Actin filaments have an inert minus end and a growing plus end to which new monomers are added. G-actin contains ATP, and this becomes hydrolyzed to ADP shortly after addition to the filament. As with tubules, a rapidly growing filament will bear an ATP cap that stabilizes the plus end. Microfilaments are often found to undergo "treadmilling" such that monomers are continuously added to the plus end and removed from the minus end while leaving the filament at the same overall length. Microfilament polymerization is prevented by a group of drugs called cytochalasins, and existing filaments are stabilized by another group called phalloidins. Like microtubules, microfilaments have associated motor proteins that will actively migrate along the fiber. The most abundant of these is

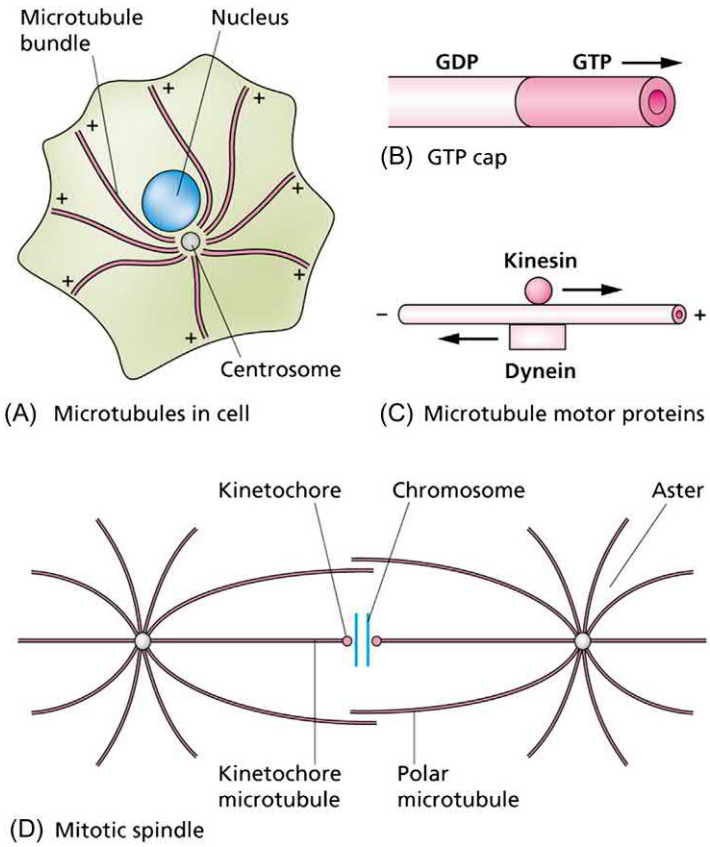

FIGURE 4.3 Microtubules: (A) arrangement in cell, (B) the GTP cap, (C) motor proteins move along the tubules, and (D) structure of the cell division spindle. *From Slack JMW. Essential developmental biology. 3rd ed. Oxford: Wiley-Blackwell; 2012.*

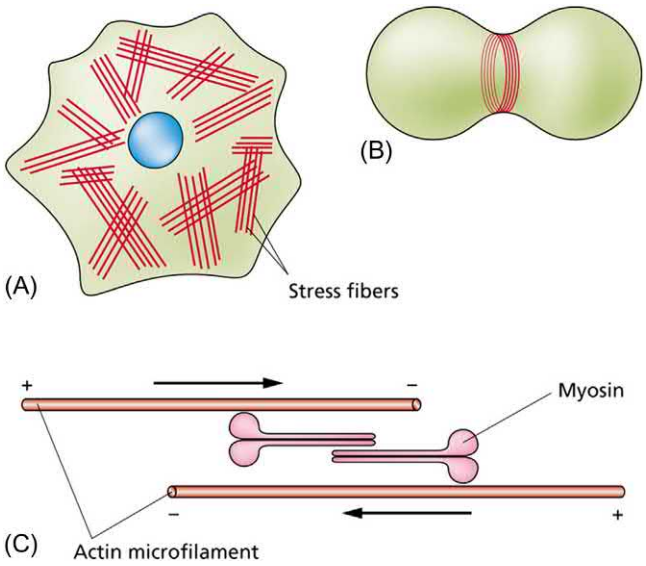

FIGURE 4.4 Microfilaments: (A) arrangement in cell, (B) role in cell division, and (C) contraction achieved by movement of myosin along microfilament. *From Slack JMW. Essential developmental biology. 3rd ed. Oxford: Wiley-Blackwell; 2012.*

myosin II, which moves toward the plus end of microfilaments, the process being driven by the hydrolysis of ATP. To bring about contraction of a filament bundle the myosin is assembled as short bipolar filaments with motile centers at both ends. If neighboring actin filaments are arranged with opposite orientation then the motor activity of the myosin will draw the filaments past each other leading to a contraction of the filament bundle.

Microfilaments can be arranged in various different ways depending on the nature of the accessory proteins with which they are associated. Contractile assemblies contain microfilaments in antiparallel orientation associated with myosin. These are found in the contractile ring that is responsible for cell division and in the stress fibers by which fibroblasts exert traction on their substratum. Parallel bundles are found in filopodia and other projections from the cell. Gels composed of short randomly orientated filaments are found in the cortical region of the cell.

Small GTPases

There are three well-known GTPases that activate cell movement in response to extracellular signals: RHO,

RAC, and CDC42. They are activated by numerous tyrosine kinase-, G-coupled-, and cytokine-type receptors. Activation involves exchange of GDP for GTP and many downstream proteins can interact with the activated forms. RHO normally activates the assembly of stress fibers. RAC activates the formation of lamellipodia and ruffles. CDC42 activates formation of filopodia. In addition all three promote the formation of focal adhesions, which are integrin-containing junctions to the extracellular matrix (ECM). These proteins can also affect gene activity through the kinase cascade signal transduction pathways.

The cell surface

The plasma membrane is the frontier between the cell and its surroundings. It is a phospholipid bilayer incorporating many specialized proteins. Very few substances are able to enter and leave cells by simple diffusion; in fact, this method is really only available to low molecular weight hydrophobic molecules such as retinoic acid, steroids, or thyroid hormones. The movement of inorganic ions across the membrane is very tightly controlled. The main control is exerted by a sodium−potassium exchanger known as the NaKATPase that expels sodium (Na) and imports potassium (K) ions. Because three sodium ions are expelled for every two potassium ions imported, this process generates an electric potential difference across the membrane, which is intensified by differential back diffusion of the ions. The final membrane potential is negative inside the cell and ranges from about 10 mV in red blood cells to about 70 mV in excitable cells such as neurons. This maintenance of membrane potential by the NaKATPase accounts for a high fraction of total cellular ATP consumption. Calcium (Ca) ions are very biologically active within the cell and are normally kept at a very low intracellular concentration, about 10^{-7} M. This is about 10^4 times lower than the typical exterior concentration, which means that any damage to the plasma membrane is likely to let in a large amount of calcium which will damage the cell beyond repair. The proteins of the plasma membrane are sometimes very hydrophobic molecules entirely contained within the lipid phase, but more usually they have hydrophilic regions projecting to the cell exterior or to the interior cytoplasm or both. These proteins have a huge range of essential functions. Some are responsible for anchoring cells to the substrate or to other cells through adhesion molecules and junctional complexes. Others, including ion transporters and carriers for a large range of nutrients, are responsible for transporting molecules across the plasma membrane. Then there are the receptors for extracellular signaling molecules (hormones, neurotransmitters, and growth factors) which are critical for controlling cellular properties and behavior. Some types of receptor serve as ion channels, for example, admitting a small amount of calcium when stimulated by their specific ligand. Other types of receptor are enzymes and initiate a metabolic cascade of intracellular reactions when stimulated. These reaction pathways often involve protein phosphorylation and frequently result in the activation of a transcription factor and thereby the expression of specific target genes. The repertoire of responses that a cell can show depends on which receptors it possesses, how these are coupled to signal transduction pathways, and how these pathways are coupled to gene regulation. It is sometimes called the "competence" of the cell. The serum that is usually included in tissue culture media contains a wide range of hormones and growth factors and is itself likely to stimulate many of the cell surface receptors.

Cell adhesion molecules

Organisms are not just bags of cells, rather each tissue has a definite cellular composition and microarchitecture. This is determined partly by the cell surface molecules by which cells interact with each other and partly by the components of the ECM. Virtually all proteins on the cell surface or in the ECM are glycoproteins, containing oligosaccharide groups added before secretion from the cell. These carbohydrate groups often have rather little effect on the biological activity of the protein, but they may affect its physical properties and stability.

Cells are attached to each other by adhesion molecules (Fig. 4.5). Among these are the cadherins that stick cells together in the presence of Ca, the cell adhesion molecules (CAMs) that do not require Ca, and the integrins that attach cells to the ECM. When cells come together, they often form gap junctions at the region of contact. These consist of small pores joining the cytosol of the two cells. The pores, or connexons, are assembled from proteins called connexins and can pass molecules up to about 1000 molecular weight by passive diffusion.

Cadherins are a family of single-pass transmembrane glycoproteins that can adhere tightly to similar molecules on other cells in the presence of calcium. They are the main factors attaching embryonic cells together, which is why embryonic tissues can often be caused to disaggregate simply by removal of calcium. The cytoplasmic tail of cadherins is anchored to actin bundles in the cytoskeleton by a complex including proteins called catenins. One of these, β-catenin, is also a component of the important WNT signaling pathway, providing a link between cell signaling and cell association. Cadherins were first named for the tissues in which they were originally found, so E-cadherin occurs mainly in epithelia and N-cadherin occurs mainly in neural tissue.

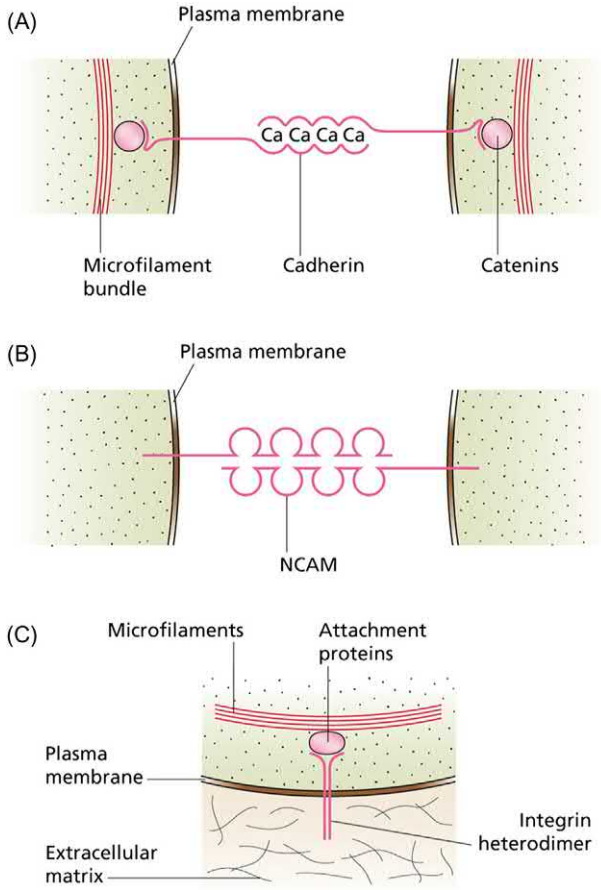

FIGURE 4.5 Cell adhesion molecules: (A) calcium-dependent system, (B) calcium-independent system, and (C) adhesion to the extracellular matrix. *From Slack JMW. Essential developmental biology. 3rd ed. Oxford: Wiley-Blackwell; 2012.*

The immunoglobulin superfamily is made up from single-pass transmembrane glycoproteins with a number of disulfide-bonded loops on the extracellular region, similar to the loops found in antibody molecules. They also bind to similar molecules on other cells, but unlike the cadherins, they do not need calcium ions to do so. The neural CAM is composed of a large family of different proteins formed by alternative splicing. It is most prevalent in the nervous system but also occurs elsewhere. It may carry a large amount of polysialic acid on the extracellular domain, and this can inhibit cell attachment because of the repulsion between the concentrations of negative charge on the two cells.

The integrins are cell surface glycoproteins that interact mainly with components of the ECM. They are heterodimers of α and β subunits and require either magnesium or calcium ions for binding. There are numerous different α and β chain types, and so there is a very large number of potential heterodimers. Integrins are attached by their cytoplasmic domains to microfilament bundles, so, such as cadherins, they provide a link between the outside world and the cytoskeleton. They are also thought on occasion to be responsible for the activation of signal transduction pathways and new gene transcription following exposure to particular ECM components.

Extracellular matrix

Glycosaminoglycans (GAGs) are unbranched polysaccharides composed of repeating disaccharides of an amino sugar and a uronic acid, usually substituted with some sulfate groups. GAGs are constituents of proteoglycans, which have a protein core to which the GAG chains are added in the Golgi apparatus before secretion. One molecule of a proteoglycan may carry more than one type of GAG chain. GAGs have a high negative charge, and a small amount can immobilize a large amount of water into a gel. Important GAGs, each of which has different component disaccharides, are heparan sulfate, chondroitin sulfate, and keratan sulfate. Heparan sulfate, closely related to the anticoagulant heparin, is particularly important for cell signaling as it is required to present various growth factors, such as the fibroblast growth factors, to their receptors. Hyaluronic acid differs from other GAGs because it occurs free and not as a constituent of a proteoglycan. It consists of repeating disaccharides of glucuronic acid and N-acetyl glucosamine and is not sulfated. It is synthesized by enzymes at the cell surface and is abundant in early embryos.

Collagens are the most abundant proteins by weight in most animals. The polypeptides, called α chains, are rich in proline and glycine. Before secretion, three α chains become twisted around each other to form a stiff triple helical structure. In the ECM the triple helices become aggregated together to form the collagen fibrils often visible in the electron microscope. There are many types of collagen, which may be composed of similar or of different α chains in the triple helix. Type I collagen is the most abundant and is a major constituent of most extracellular material. Type II collagen is found in cartilage and in the notochord of vertebrate embryos. Type IV collagen is a major constituent of the basal lamina underlying epithelial tissues. Collagen helices may become covalently crosslinked through their lysine residues, and this contributes to the changing mechanical properties of tissues with age. Elastin is another extracellular protein with extensive intermolecular cross-linking. It confers the elasticity on tissues in which it is abundant and also has some cell-signaling functions.

Fibronectin is composed of a large disulfide-bonded dimer. The polypeptides contain regions responsible for binding to collagen, to heparan sulfate, and to integrins on the cell surface. These latter, cell-binding, domains are

characterized by the presence of the amino acid sequence Arg-Gly-Asp. There are many different forms of fibronectin produced by alternative splicing. Laminin is a large extracellular glycoprotein found particularly in basal laminae. It is composed of three disulfide-bonded polypeptides joined in a cross shape. It carries domains for binding to type IV collagen, heparan sulfate, and another matrix glycoprotein, entactin.

Signal transduction

Lipid soluble molecules such as steroid hormones can enter cells by simple diffusion. Their receptors, called nuclear hormone receptors, are multidomain molecules that also function as transcription factors. Binding of the ligand activates the factor, in many cases also causing translocation to the nucleus where the receptor–ligand complex can upregulate its target genes (Fig. 4.6A). Most signaling molecules are proteins which cannot diffuse across the plasma membrane and so work by binding to specific cell surface receptors. There are three main classes of these: enzyme-linked receptors, G-protein-linked receptors, and ion channel receptors. Enzyme-linked receptors are often tyrosine kinases or Ser/Thr kinases (Fig. 4.6B). All have a ligand-binding domain on the exterior of the cell, a single transmembrane domain, and the enzyme active site on the cytoplasmic domain. For receptor tyrosine kinases, the ligand binding brings about dimerization of the receptor, and this results in an autophosphorylation, whereby each receptor molecule phosphorylates and activates the other. The phosphorylated receptors can then activate a variety of targets. Many of these are transcription factors that are activated by phosphorylation and move to the nucleus where they upregulate their target genes. In other cases there is a cascade of kinases that activate each other down the chain, culminating in the activation of a transcription factor. Roughly speaking, each class of signaling molecules has its own associated receptors and a specific signal transduction pathway; however, different receptors may be linked to the same signal transduction pathway, or one receptor may feed into more than one pathway. The effect of one pathway upon the others is often called "cross talk." The significance of cross talk can be hard to assess from biochemical analysis alone but is easier using genetic experiments in which individual components are mutated to inactivity and the overall effect on the cellular behavior can be assessed.

There are several classes of G-protein-linked receptor (Fig. 4.6C). The best known are seven-pass membrane proteins, meaning that they are composed of a single polypeptide chain crossing the membrane seven times. These are associated with trimeric G proteins composed of α, β, and γ subunits. When the ligand binds, the activated receptor causes exchange of GDP bound to the α subunit for GTP, then the activated α subunit is released and can interact with other membrane components. The most common target is adenylyl cyclase that converts ATP to cyclic adenosine monophosphate

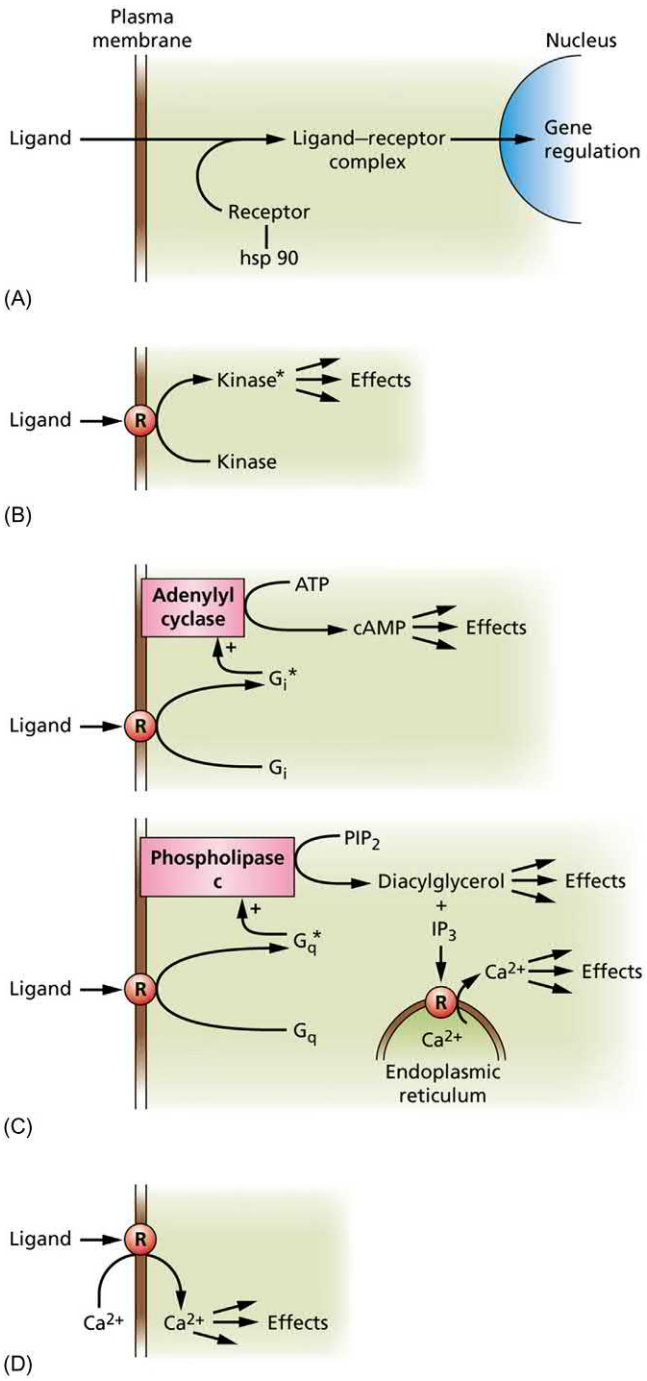

FIGURE 4.6 Different types of signal transduction: (A) nuclear receptor, (B) enzyme-linked receptor, (C) G protein–linked receptor, and (D) ion channel receptor. *From Slack JMW. Essential developmental biology. 3rd ed. Oxford: Wiley-Blackwell; 2012.*

(cAMP). cAMP activates protein kinase A (PKA) that phosphorylates various further target molecules affecting both intracellular metabolism and gene expression. Another large group of G-protein-linked receptors use a different trimeric G protein to activate the inositol phospholipid pathway (Fig. 4.6C). Here the G protein activates phospholipase C which breaks down phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol trisphosphate (IP_3). The DAG activates an important membrane-bound kinase, protein kinase C. Like PKA, this has a large variety of possible targets in different contexts and can cause both metabolic responses and changes in gene expression. The IP_3 binds to an IP_3 receptor (IP_3R) in the endoplasmic reticulum and opens Ca channels which admit Ca ions into the cytoplasm. Normally cytoplasmic calcium is kept at a very low concentration of around 10^{-7} M. An increase caused either by opening of an ion channel in the plasma membrane, or as a result of IP_3 action, can have a wide range of effects on diverse target molecules. Ion channel receptors (Fig. 4.6D) are also very important. They open on stimulation to allow passage of Na, K, Cl, or Ca ions. As mentioned above, Na and K ions are critical to the electrical excitability of nerve or muscle, and Ca ions are very potent, having a variety of effects on cell structure and function at low concentration.

Growth and death

Tissue engineering inevitably involves the growth of cells in culture, so the essentials of cell multiplication need to be understood. A typical animal cell cycle is shown in Fig. 4.7 and some typical patterns of cell division in Fig. 4.8. The cell cycle is conventionally described as consisting of four phases. M indicates the phase of mitosis (cell division), S indicates the phase of DNA replication, and G1 and G2 are the intervening growth phases. For growing cells, the increase in mass is continuous around the cycle and so is the synthesis of most of the cell's proteins. Normally the cell cycle is coordinated with the growth of mass. If it were not, cells would increase or decrease in size with each division. There are various internal controls built into the cycle, for example to ensure that mitosis does not start before DNA replication is completed. These controls operate at checkpoints around the cycle at which the process stops unless the appropriate conditions are fulfilled. Control of the cell cycle depends on a metabolic oscillator comprising a number of proteins called cyclins and a number of cyclin-dependent protein kinases (Cdks). In order to pass the M checkpoint and enter mitosis, a complex of cyclin and Cdk (called M-phase promoting factor, MPF) has to be activated. This phosphorylates and thereby activates the various components required for mitosis (nuclear breakdown, spindle formation, chromosome condensation). Exit from M phase requires the inactivation of MPF, via the destruction of cyclin. Passage of the G1 checkpoint depends on a similar process operated by a different set of cyclins and Cdks, whose active complexes phosphorylate and activate the enzymes of DNA replication. This is also the point at which the cell size is assessed. The cell cycle of G1, S, G2, and M phases is universal, although there are some modifications in special circumstances. The rapid cleavage cycles of early development have short or absent G1 and G2 phases, and there is no size check, the cells halving in volume with each division. The meiotic cycles that generate sperm and eggs for reproduction require the same active MPF complex to get through the two nuclear divisions, but there is no S phase in between. In the mature organism most cells are quiescent unless, they are stimulated by growth factors. In the absence of growth factors, cells enter a state called G0, in which the Cdks and cyclins are absent. Restitution of growth factors induces the resynthesis of these proteins and the resumption of the cycle starting from the G1 checkpoint. One factor maintaining the G0 state is a protein called Rb (retinoblastoma protein). This becomes phosphorylated, and hence deactivated, in the presence of growth factors. In the absence of Rb a transcription factor called E2F becomes active and initiates a cascade of gene expression culminating in the resynthesis of cyclins, Cdks, and other components needed to initiate S phase. Cells often have the capability for exponential growth in tissue culture (Fig. 4.8C), but this is very rarely found in animals. Although some differentiated cell types can go on dividing, there is a general tendency for differentiation to be accompanied by a slow down or cessation of division. In postembryonic life, most cell division is found among stem cells and their immediate progeny called transit amplifying cells. Stem cells are cells that can both reproduce themselves and generate differentiated progeny for their particular tissue type (Fig. 4.8D). This does not necessarily mean that every division of a stem cell has to be an asymmetrical one, but over a period of time, half the progeny will go to renewal and half to differentiation. The term "stem cell" is also used for embryonic stem cells of early mammalian embryos. These are early embryo-type cells that can be grown in culture and are capable of repopulating embryos and contributing to all tissue types.

Asymmetric cell divisions necessarily involve the segregation of different cytoplasmic determinants to the two daughter cells, evoking different patterns of gene activity in their nuclei and thus bringing about different pathways of development. The asymmetry often arises from autosegregation of a self-organizing protein complex called the PAR complex. This is active both for asymmetric division in early embryos and for those in various tissues in postnatal life.

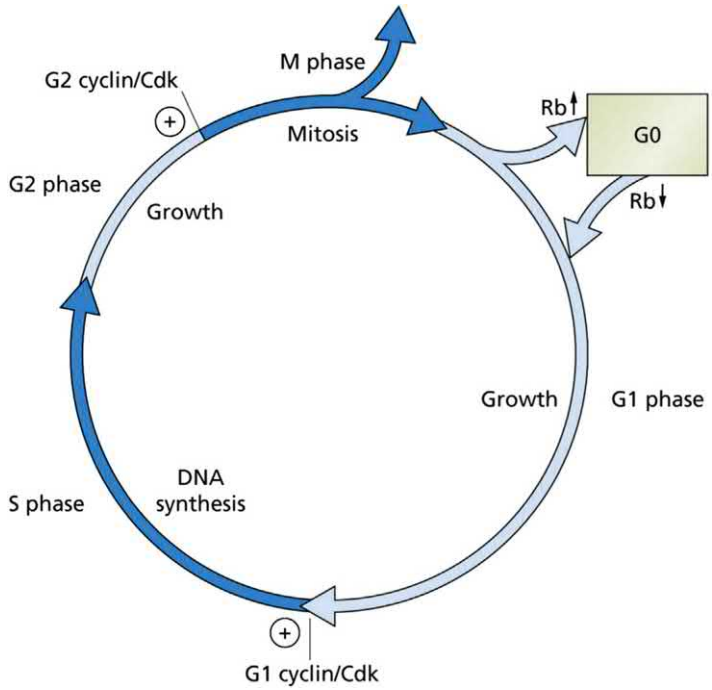

FIGURE 4.7 The cell cycle, with phases of growth, DNA replication and division. *From Slack JMW. Essential developmental biology. 3rd ed. Oxford: Wiley-Blackwell; 2012.*

Culture media

Tissue engineering is heavily focused on the growth of cells, tissues, and even organs in vitro, so it is very important to understand the principles of cell culture (Fig. 4.9).

On a laboratory scale, cells are usually grown in small containers made of polystyrene, which is optically clear and easily sterilized by irradiation. To enable attachment of cells the plastic needs to be specially treated. For specific application a layer of fibronectin or collagen or polylysine may be added to further improve cell attachment. Some cell types can be grown in suspension culture often in bottles incorporating a rotating stirrer arm. Since such bottles may be nearly filled with medium, they allow for large amounts of cell production relative to similar-sized surface culture methods. But for visual observation a sample must be taken out of the vessel for examination. For really large-scale cell production a bioreactor must be used. This is a temperature controlled vessel with controlled flows of medium and gases and continuous monitoring of conditions. Massive bioreactors are used in the pharmaceutical industry for the production of antibodies or recombinant proteins from mammalian cell cultures.

Mammalian cells will only remain in good condition very close to the normal body temperature, so good temperature control is essential during culture. Because water can pass across the plasma membranes of animal cells, the medium must match the osmolarity of the cell interior; otherwise cells will swell or shrink due to osmotic pressure difference. Mammalian cell media generally have a total osmolarity about 350 mOsm. The pH needs to be tightly controlled, 7.4 being normal. The pH control is usually achieved with bicarbonate-CO_2 buffers (2.2 g/L bicarbonate and 5% CO_2 being a common combination). These give better results with most animal cells than other buffers, perhaps because bicarbonate is also a type of nutrient. Most media contain phenol red indicator. This has a red color at neutral pH and goes purple at alkaline pH and yellow at acid pH. The medium must contain a variety of components: salts, amino acids, sugars, plus low levels of specific hormones, and growth factors required for the particular cell type in question. Because of the complexity of tissue culture media, they are rarely optimized for a given purpose by varying every one of the components systematically. Usually changes are incremental and the result of a "gardening" type of approach rather than a systematic one. The requirement for hormones and growth factors is usually met by including some animal serum, often 10% fetal calf serum. This is long-standing practice but has two substantial disadvantages. Serum can never be completely characterized, and there are often differences between batches of serum that can be critical for experimental results. Also animal serum cannot now be used for the preparation of cells that are intended for implantation into human patients. This is because of the perceived possibility of transmitting animal diseases to patients. The various problems with serum have led to an increased formulation of serum-free media, which are often completely "defined" meaning that all the macromolecular hormones, growth factors, and other components in the medium are chemically pure.

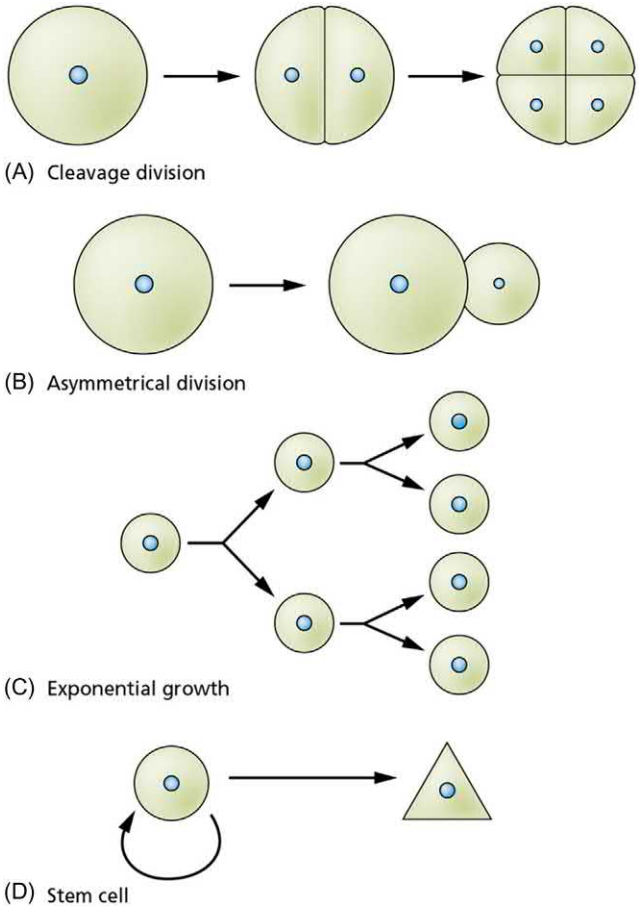

FIGURE 4.8 Types of cell division: (A) cleavage as found in early embryos, (B) asymmetrical division also found in some early embryos, (C) exponential growth found in tissue culture, and (D) stem-cell division found in renewal tissues in animals. *From Slack JMW. Essential developmental biology. 3rd ed. Oxford: Wiley-Blackwell; 2012.*

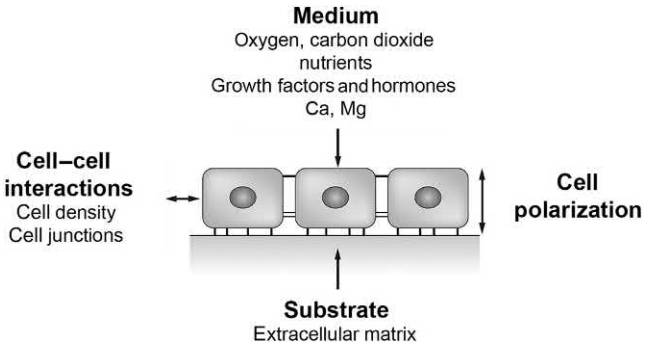

FIGURE 4.9 Multiple factors control the cellular environment for cells in culture.

Assuming cells are kept in near optimal conditions, they can in principle grow exponentially, with a constant doubling time. Indeed, it is possible to grow many types of tissue culture cell in exponential cultures rather like microorganisms. In order to keep them growing at maximal rate, they need to have their medium renewed regularly and to be subcultured and replated at lower density whenever they approach confluence, that is, approach a density at which they cover all the available surface. Subculturing is usually carried out by treatment with the enzyme trypsin, which degrades much of the extracellular and cell surface protein and makes the cells drop off the substrate and become roughly spherical bodies in suspension. The trypsin is diluted out, and the cells are transferred at lower density into new flasks. The cells take an hour or two to resynthesize their surface molecules and can then adhere to the new substrate and carry on growing.

Cells in tissues and organs

Cell types

On the basis of light microscopy, it is estimated that there are about 210 different types of differentiated cell in the mammalian body. This number is certainly an underestimate since there are many subtypes of cells that cannot be seen via the light microscope, particularly the different types of neuron in the nervous system and different types of lymphocyte in the immune system. Cells types are different from one another because they are expressing different subsets of genes and hence contain different proteins. The products of a relatively small number of genes may dominate the appearance of a differentiated cell, for example, the proteins of the contractile apparatus of skeletal muscle are very abundant. However, a typical cell will express many thousands of genes, and its character will also depend on the genes expressed at low levels and, by exclusion, on the genes that are not expressed. It is possible to control cell differentiation to some extent. There are special culture media that are favorable for differentiation of particular cell types, such as adipocytes, muscle, or bone. In some cases differentiated cells can continue to grow in pure culture, but in many cases, differentiation causes slowing or cessation of cell division.

From a morphological point of view, most cells can be regarded as epithelial or mesenchymal (Fig. 4.10). These terms relate to cell shape and behavior rather than to embryonic origin. An epithelium is a sheet of cells, arranged on a basement membrane, each cell joined to its neighbors by specialized junctions, and showing a distinct apical-basal polarity. Mesenchyme is a descriptive term for scattered stellate-shaped cells embedded in loose ECM. It fills up much of the space within the embryo and later forms fibroblasts, adipose tissue, smooth muscle, and skeletal tissues.

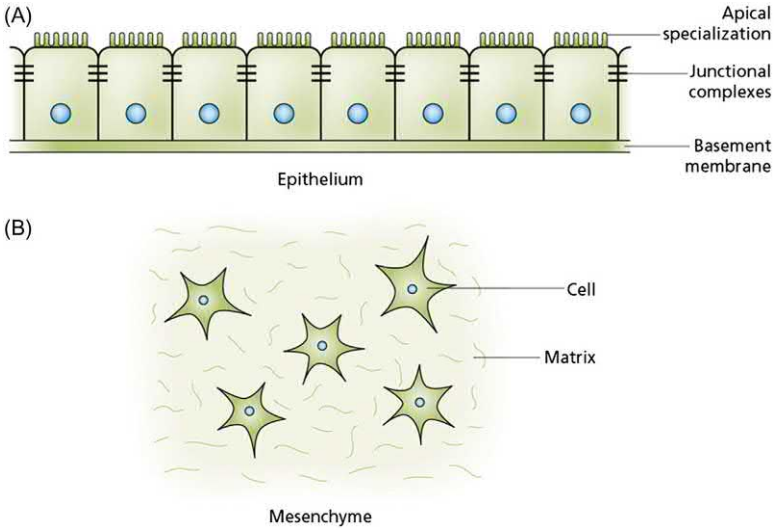

FIGURE 4.10 Most tissues are composed of epithelial (A) and mesenchymal (B) components. *From Slack JMW. Essential developmental biology. 3rd ed. Oxford: Wiley-Blackwell; 2012.*

Tissues

From a developmental biology standpoint, a tissue is the set of cell types arising from one sort of progenitor or stem cell. For example, the intestinal epithelium is a tissue. It is structured into crypts, containing undifferentiated cells, and villi covered with differentiated cells. There are four principal mature cell types: absorptive, goblet, Paneth, and enteroendocrine cells. The stem cells are located at the crypt base and produce progenitor cells, called transit amplifying cells, which divide a few times before differentiating into one of the four mature cell types. The tissue comprises the stem cells, the transit amplifying cells, and the differentiated cells. This is an example of a renewal tissue, in which there is continuous production and replacement of differentiated cells. Not all tissues are renewal tissues, although most display a small degree of renewal or the ability to replace the constituent cell types following damage. For example, the central nervous system arises from the neuroepithelium of the early embryo and contains a variety of types of neuron together with glial cells, especially astrocytes and oligodendrocytes. There is only replacement of neurons in two regions of the brain and turnover of glial populations is slow. But because it arises from a clearly identified progenitor cell population, the central nervous system can be regarded as one tissue.

In cases where differentiated cells are derived from different stem or progenitor cell populations, they cannot be considered as a single tissue. For example, the thyroid gland contains many follicles of thyroglobulin-producing epithelial cells. It also contains endocrine cells producing the hormone calcitonin. However, the latter do not arise from the same endodermal bud as the rest of the thyroid. Instead they arise from the neural crest of the embryo. Likewise, many tissues contain macrophages (histiocytes) derived from the hematopoietic stem cells of the bone marrow. These cells are properly considered to belong to the blood/immune tissue rather than the epithelia or connective tissues in which they reside.

Organs

Organs are the familiar structures in the body, which are each associated with a particular function. They are typically composed of several tissue layers and arise from multiple developmental origins. For example, the stomach has the function of preliminary food digestion. The lining of the stomach is one tissue, the gastric epithelium, derived from gastric stem cells. The outer layers consist of smooth muscle and connective tissues, blood vessels, nerve fibers, and cells of the blood and immune system. Together, these make up a discrete and integrated body part: an organ.

Likewise, a single named muscle is one organ, composed of several tissues, each consisting of more than one cell type. A muscle contains many muscle fibers that are postmitotic multinucleate cells. It also contains muscle satellite cells that are stem cells that can regenerate fibers. The fibers and the satellite cells are one tissue, arising from the myotome of the somites of the embryo. Surrounding the bundles of fibers are connective tissue sheaths composed of fibroblasts and ECM derived from lateral plate mesoderm, together with blood vessels, nerve fibers, and cells of the blood and immune system.

Organs often have an epithelial and mesenchymal component. The epithelium is usually the functional part, for example, the epithelial lining of the various segments of the gut have specific properties of protection, absorption or secretion, while the underlying mesenchyme (often called “stroma” in adult organs) provides mechanical

support, growth factors, and physiological response in terms of muscular movements.

With the exception of the kidney, all other organs draw their epithelium and mesenchyme from different germ layers of the embryo. The implication of this for tissue engineering is that it will probably be necessary to assemble structures from separate epithelial and mesenchymal cells, designed such that each population can support the other by secretion of appropriate factors. A further consideration for the creation of organs in vitro is that cells need a continuous supply of nutrients and oxygen and continuous removal of waste products. In vivo this is achieved by means of the blood vascular system that culminates in capillary beds of enormous density such that all cells are within a few cell diameters of the blood. For tissue engineering the lesson is clear: it is possible to grow large avascular structures only so long as they are two dimensional: for example, large sheets of epidermis a few cells thick can be grown in vitro and used successfully for skin grafting. But any tissue more than a fraction of a millimeter thickness needs to be provided with some sort of vascular system.

Tissue engineering needs not attempt to copy everything that is found in the normal body. However, it is necessary to be aware of the constraints provided by the molecular biology of the cell. Factors to be considered include the following:

- How to keep cells in the desired state by providing the correct substrate and medium?
- How to create engineered structures containing two or more cell types of different origins which can sustain one another?
- How to provide a vascular system capable of delivering nutrients and removing waste products?
- How to control cell division to achieve homeostasis rather than uncontrolled growth or regression?

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Chapter 5

Molecular organization of cells

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Introduction

Multicellular tissues exist in one of two types of cellular arrangements, epithelial or mesenchymal. Epithelial cells adhere tightly to each other at their lateral surfaces and to an organized extracellular matrix (ECM) at their basal domain, thereby producing a sheet of cells resting on a basal lamina with an apical surface. Mesenchymal cells, in contrast, are individual cells with a bipolar morphology that are held together as a tissue within a three-dimensional (3D) ECM (Fig. 5.1). The conversion of epithelial cells into mesenchymal cells, an "epithelial−mesenchymal transition" (EMT), is central to many aspects of embryonic morphogenesis and adult tissue repair, as well as a number of disease states [1−3]. The reverse process whereby mesenchymal cells coalesce into an epithelium is a "mesenchymal−epithelial transition" (MET). Understanding the molecules that regulate this transition between epithelial and mesenchymal states offers important insights into how cells and tissues are organized.

The early embryo is structured as one or more epithelia. An EMTallows the rearrangements of cells to create additional morphological features. Well-studied examples of EMTs during embryonic development include gastrulation in *Drosophila* [3], the emigration of primary mesenchyme cells (PMCs) in sea urchin embryos [4], and gastrulation in amniotes (reptiles, birds, and mammals) at the primitive streak [2]. EMTs also occur later in vertebrate development, such as during the emigration of neural crest cells from the neural tube [5], the formation of the sclerotome from epithelial somites, and palate fusion [2]. The reverse process of MET is likewise crucial to development; examples include the condensation of mesenchymal cells to form the notochord and somites [6], kidney tubule formation from nephrogenic mesenchyme [7], and the creation of heart valves from cardiac mesenchyme [8]. In the adult organism, EMTs and METs occur during wound healing and tissue remodeling [9]. The conversion of neoplastic epithelial cells into invasive cancer cells has long been considered an EMT process [1,10]. However, there are also examples of tumor cells that have functional cell−cell adhesion junctions yet are still migratory and invasive as a group [11]. This "collective migration" also occurs during development [11]. Hence, there is debate whether an EMT model accurately describes all epithelial metastatic cancers. Similarly, the fibrosis of cardiac, kidney, lens, and liver epithelial tissue has also long been categorized as an EMT event [6,12]. However, research in the kidney in vivo shows that the myofibroblasts induced after kidney injury are derived from mesenchymal pericytes rather than the proximal epithelial cells [13]. Therefore, defining the origin of the cells that contribute to fibrotic tissue scarring (epithelial or otherwise) will require further investigation.

The focus of this chapter is on the molecules that regulate the organization of cells into epithelium or mesenchyme. We will first discuss the cellular changes that occur during an EMT, including changes in cell−cell and cell−ECM adhesions, changes in cell polarity, and the stimulation of invasive cell motility. Then we will consider the molecules and mechanisms that control the EMT or MET, including the structural molecules, transcription factors, and signaling pathways that regulate EMTs.

Molecules that organize cells

The conversion of an epithelial sheet into individual migratory cells and back again requires the coordinated changes of many distinct families of molecules.

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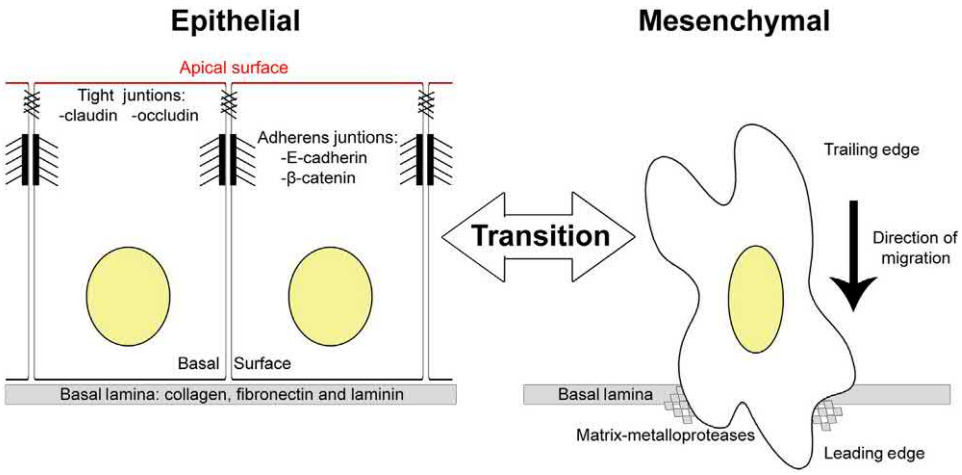

FIGURE 5.1 **Epithelial versus mesenchymal.** Epithelial cells adhere tightly together by tight junctions and adherens junctions localized near the apical surface. Epithelial cells also have a basal surface that rests on a basal lamina. In contrast, mesenchymal cells do not have well-defined cell−cell adhesion complexes; they have front-end/back-end polarity instead of apicobasal polarity, and mesenchymal cells are characterized by their ability to invade the basal lamina.

Changes in cell−cell adhesion

Epithelial cells are held together by specialized cell−cell junctions, including adherens junctions, desmosomes, and tight junctions [14]. These junctions are localized in the lateral domain near the apical surface and establish the apical polarity of the epithelium. For an epithelial sheet to produce individual mesenchymal cells, cell−cell adhesions must be disrupted. The principal transmembrane proteins that mediate cell−cell adhesions are members of the cadherin superfamily [15]. E-cadherin and N-cadherin are classical cadherins that interact homotypically through their extracellular immunoglobulin G domains with like-cadherins on adjacent cells. Cadherins are important mediators of cell−cell adhesion. For example, misexpression of E-cadherin is sufficient to promote cell−cell adhesion and assembly of adherens junctions in fibroblasts [16]. In epithelial cancers (carcinomas), E-cadherin acts as a tumor suppressor [10]. In a mouse model for β-cell pancreatic cancer, the loss of E-cadherin is the rate-limiting step for transformed epithelial cells to become invasive [17]. Although the loss of cadherin-mediated cell−cell adhesion is necessary for an EMT, the loss of cadherins is not always sufficient to generate a complete EMT in vivo. For example, the neural tube epithelium in mice expresses N-cadherin, but in the N-cadherin knockout mouse an EMT is not induced in the neural tube [18]. Hence, cadherins are essential for maintaining epithelial integrity, and the loss of cell−cell adhesion caused by the reduction of cadherin function is an important step for an EMT.

One characteristic of an EMT is "cadherin switching." Often, epithelia that express E-cadherin will downregulate E-cadherin expression at the time of the EMT and express different cadherins such as N-cadherin [19]. Cadherin switching may promote motility. For instance, in mammary epithelial cell lines, the misexpression of N-cadherin is sufficient for increased cell motility. However, blocking N-cadherin expression does not result in motility even though the adherens junctions are reduced. Hence, cadherin switching may be necessary for cell motility, but cadherin switching alone is not sufficient to bring about a complete EMT [20].

There are several ways in which cadherin expression and function are regulated. Transcription factors that are central to most EMTs, such as Snail-1, Snail-2, Zinc finger E-box-binding (Zeb)1, Zeb2, Twist, and E2A, all bind to E-boxes on the *E-cadherin* promoter and repress the transcription of *E-cadherin* [21]. Posttranscriptionally, the E-cadherin protein is ubiquitinated by the E3-ligase, Hakai, which targets E-cadherin to the proteasome [22]. E-cadherin turnover at the membrane is regulated by either caveolae-dependent endocytosis or clathrin-dependent endocytosis [23], and p120-catenin prevents endocytosis of E-cadherin at the membrane [24]. E-cadherin function can also be disrupted by matrix metalloproteases, which degrade the extracellular domain of E-cadherin [25]. Some or all of these mechanisms may occur during an EMT to disrupt cell−cell adhesion.

Cell−cell adhesion is maintained principally by cadherins, and changes in cadherin expression are typical of an EMT.

Changes in celleextracellular matrix adhesion

Altering the way in which a cell interacts with the ECM is also important in EMTs. For example, at the time that sea urchin PMCs ingress, the cells have increased

adhesiveness for ECM [4]. CelleECM adhesion is mediated principally by integrins. Integrins are transmembrane proteins composed of two noncovalently linked subunits, α and β, that bind to ECM components such as fibronectin, laminin, and collagen. The cytoplasmic domain of integrins links to the cytoskeleton and interacts with signaling molecules. Changes in integrin function are required for many EMTs, including neural crest emigration [26], mouse primitive streak formation [2], and cancer metastasis [27]. However, the misexpression of integrin subunits is not sufficient to bring about a full EMT in vitro [28] or in vivo [29].

The presence and function of integrins are modulated in several ways. For example, the promoter of the *integrin β6* gene is activated by the transcription factor Ets-1 during colon carcinoma metastasis [30]. Most integrins can also cycle between "on" (high-affinity) or "off" (low-affinity) states. This "inside-out" regulation of integrin adhesion occurs at the integrin cytoplasmic tail [31]. In addition to integrin activation, the "clustering" of integrins on the cell surface affects the overall strength of integrineECM interactions. The increased adhesiveness of integrins caused by clustering, known as avidity, can be activated by chemokines and depends on RhoA and phosphatidylinositol 3° kinase (PI3K) activity [31].

Changes in ECM adhesion are required for an EMT. Cell−ECM adhesions are maintained by integrins, which have varying degrees of adhesiveness depending on the presence, activity, or avidity of the integrin subunits.

Changes in cell polarity and stimulation of cell motility

Cellular polarity is defined by the distinct arrangement of cytoskeletal elements and organelles in epithelial versus mesenchymal cells. Epithelial polarity is characterized by cell−cell junctions found near the apicolateral domain (nonadhesive surface), and a basal lamina opposite of the apical surface (adhesive surface). Mesenchymal cells, in contrast, do not have apicobasal polarity, but rather front-end/back-end polarity, with actin-rich lamellipodia and Golgi localized at the leading edge [2]. Molecules that establish cell polarity include Cdc42, PAK1, PI3K, PTEN, Rac, Rho, and the PAR proteins [32,33]. Changes in cell polarity help to promote an EMT. In mammary epithelial cells, the activated transforming growth factor-β (TGF-β) receptor II causes Par6 to activate the E3 ubiquitin ligase Smurf1, which then targets RhoA to the proteasome. The loss of RhoA activity results in the loss of cell−cell adhesion and epithelial cell polarity [34].

For mesenchymal cells to leave the epithelium, they must become motile. Many of the same polarity (Crumbs, PAR, and Scribble complexes), structural (actin and microtubules), and regulatory molecules (Cdc42, Rac1, and RhoA) that govern epithelial polarity are also central to cell motility [35]. Cell motility mechanisms also vary depending on whether the environment is two-dimensional or 3D [36]. Many mesenchymal cells express the intermediate filament vimentin, which may be responsible for several aspects of the EMT phenotype [37].

In short, a wide variety of structural, polarity, and regulatory molecules must be reassigned as cells transition between epithelial polarity and mesenchymal migration.

Invasion of the basal lamina

In most EMTs, the emerging mesenchymal cells must penetrate a basal lamina, which consists of ECM components such as collagen type IV, fibronectin, and laminin. The basal lamina functions to stabilize the epithelium and is a barrier to migratory cells [38]. One mechanism that mesenchymal cells use to breach the basal lamina is to produce enzymes that degrade it. Plasminogen activator is one protease associated with a number of EMTs, including neural crest emigration [38] and the formation of cardiac cushion cells during heart morphogenesis [39]. The type II serine protease TMPRSS4 also promotes an EMT and metastasis when overexpressed in vitro and in vivo [40]. Matrix-metalloprotease (MMPs) are also important for many EMTs. When MMP-2 activity is blocked in the neural crest EMT, neural crest emigration is inhibited, but not neural crest motility [41]. In mouse mammary cells, MMP-3 overexpression is sufficient to induce an EMT in vitro and in vivo [42]. Misexpressing MMP-3 in cultured cells induces an alternatively spliced form of Rac1 (Rac1b), which then causes an increase in reactive oxygen species (ROS) intracellularly, and Snail-1 expression. Either Rac1b activity or ROS is necessary and sufficient for an MMP-3einduced EMT [43]. Hence, a number of extracellular proteases are important to bring about an EMT.

Although epithelial cells undergoing an EMT eventually lose cell−cell adhesion and apicobasal polarity and gain invasive motility, the EMT program is not necessarily ordered or linear. For example, in a study in which neural crest cells were labeled with cell adhesion or polarity markers and individual live cells were observed to undergo the EMT in slice culture, neural crest cells changed epithelial polarity either before or after the complete loss of cell−cell adhesion, or lost cell−cell adhesion either before or after cell migration commenced [44]. Therefore, whereas an EMTconsists of several distinct phases, these steps may occur in different orders or combinations, some of which (e.g., the complete loss of cell-cell adhesion) may not always be necessary.

Changes in a wide range of molecules are needed for an EMT because epithelial cells lose cell–cell adhesion, change cellular polarity, and gain invasive cell motility.

The epithelial–mesenchymal transition transcriptional program

At the foundation of every EMT or MET program are the transcription factors that regulate the gene expression required for these cellular transitions. Whereas many of the transcription factors that regulate EMTs have been identified, the complex regulatory networks are still incomplete. Here we review the transcription factors that are known to promote the various phases of an EMT. Then we examine how these EMT transcription factors themselves are regulated at the promoter and posttranscriptional levels.

Transcription factors that regulate epithelial–mesenchymal transition

The Snail family of zinc-finger transcription factors, including Snail-1 and Snail-2 (formerly Snail and Slug), are direct regulators of cell–cell adhesion and motility during EMTs [21,45]. The knockout of *Snail-1* in mice is lethal early in gestation, and the presumptive primitive streak cells that normally undergo an EMT retain apicobasal polarity and adherens junctions, and express *E-cadherin* messenger RNA [46]. Snail-1 misexpression is sufficient for breast cancer recurrence in a mouse model in vivo, and high levels of *Snail-1* predict the relapse of human breast cancer [47]. Snail-2 is necessary for the chicken primitive streak and neural crest EMTs [48]. One way in which Snail-1 or Snail-2 causes decreases in cell–cell adhesion is by repressing the *E-cadherin* promoter [21]. This repression requires the mSin3A corepressor complex, histone deacetylases, and components of the Polycomb 2 complex [49]. Snail-1 is also a transcriptional repressor of the tight junction genes *Claudin* and *Occludin* [21] and the polarity gene *Crumbs3* [50]. The misexpression of Snail-1 and Snail-2 further leads to the transcription of proteins important for cell motility, such as fibronectin, vimentin [51], and RhoB [52]. Moreover, Snail-1 promotes invasion across the basal lamina. In Madin–Darby Canine Kidney (MDCK) cells, the misexpression of Snail-1 represses laminin (basement membrane) production [53] and indirectly upregulates *mmp-9* transcription [54]. Snail and Twist also make cancer cells more resistant to senescence, chemotherapy, and apoptosis, and endow cancer cells with "stem cell" properties [6]. Hence, Snail-1 or Snail-2 is necessary and sufficient for bringing about many of the steps of an EMT, including loss of cell–cell adhesion, changes in cell polarity, gain of cell motility, invasion of the basal lamina, and increased proliferation and survival.

Other zinc finger transcription factors important for EMTs are Zeb homeobox 1 (Zeb1; also known as δEF1), and Zeb2 (also known as Smad-interacting protein-1; Sip1). Both Zeb1 and Zeb2 bind to the *E-cadherin* promoter and repress transcription [21]. Zeb1 can also bind to and repress the transcription of the polarity proteins Crumbs3, Pals1-associated tight junction proteins, and Lethal giant larvae 2 [55]. Zeb2 is structurally similar to Zeb1, and Zeb2 overexpression is sufficient to downregulate E-cadherin, dissociate adherens junctions, and increase motility in MDCK cells [56].

The lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) transcription factors also have an important role in EMTs. For instance, the misexpression of Lef-1 in cultured colon cancer cells reversibly causes the loss of cell–cell adhesion [57]. LEF/TCF transcription factors directly activate genes that regulate cell motility, such as the L1 adhesion molecule [58], and the *fibronectin* gene [59]. LEF/TCF transcription factors also upregulate genes required for basal lamina invasion, including *mmp-3* and *mmp-7* [60].

Other transcription factors that have a role in promoting EMTs are the class I basic helix-loop-helix factors E2-2A and E2-2B [61], the forkhead box transcription factor FOXC2 [62], the homeobox protein Goosecoid [63], and the homeoprotein Six1 [64,65].

Transcription factors that regulate an EMT often do so by directly repressing cell adhesion and epithelial polarity molecules and by upregulating genes required for cell motility and basal lamina invasion.

Regulation at the promoter level

Given the importance of the Snail, Zeb, and LEF/TCF transcription factors in orchestrating the various phases of an EMT, it is essential to understand the upstream events that regulate these EMT-promoting transcription factors. The activation of *Snail-1* transcription in *Drosophila* requires the transcription factors Dorsal [nuclear factor κB (NF-κB)] and Twist [21]. The human *Snail-1* promoter also has functional NF-κB sites [66], and blocking NF-κB reduces *Snail-1* transcription [67]. In addition, a region of the *Snail-1* promoter is responsive to integrin-linked kinase (ILK) [21], and ILK can activate Snail-1 expression via poly-adenosine phosphate-ribose polymerase [68]. In mouse mammary epithelial cells, high-mobility group protein A2 and Smads activate *Snail-1* expression and subsequently *Snail-2*, *Twist*, and *Id2* transcription [69]. For *Snail-2* expression, myocardin-related transcription factors (MRTFs) interact with Smads to

induce *Snail-2* [70] and MRTFs may have a role in metastasis [71] and fibrosis [72]. There are also several *Snail-1* transcriptional repressors. In breast cancer cell lines, metastasis-associated protein 3 binds directly to and represses the transcription of *Snail-1* in combination with the Mi-2/nucleosome remodeling deacetylase complex [73], as does lysine-specific demethylase [74]. The Ajuba LIM proteins (Ajuba, LIMD1, and WTIP) are additional transcriptional corepressors of the Snail family [75].

The transcription of LEF/TCF genes such as *Lef-1* are activated by Smads [76]. The misexpression of Snail-1 results in the transcription of *δEF-1* and *Lef-1* through a yet unknown mechanism [21].

Posttranscriptional regulation of epithelial–mesenchymal transition transcription factors

The activity of EMT transcription factors is also regulated at the protein level, including translational control, protein stability (targeting to the proteasome), and nuclear localization. Noncoding RNAs are emerging as important regulator EMTs. In a breast cancer model, Myc activates the expression of microRNA-9 (miR-9), and miR-9 directly binds to and represses the *E-cadherin* promoter [77]. Members of the miR-200 family repress the translation of *Zeb1*, and the expression of these miR-200 family members is repressed by Snail-1. In addition, *Zeb2* transcription can be activated by naturally occurring RNA antisense transcripts [78]. It is not yet known whether there are noncoding RNAs that regulate Snail family members. However, the Y-box binding protein-1 is important for the selective activation of *Snail-1* translation [79].

Protein stability is another layer of EMT control. Snail-1 is phosphorylated by glycogen synthase kinase 3β (GSK-3β) and targeted for destruction [80]. Therefore, the inhibition of GSK-3β activity by Wnt signaling may have multiple roles in an EMT, leading to the stabilization of both β-catenin and Snail-1. Some proteins that prevent GSK 3β-mediated phosphorylation (and thus promote Snail-1 activation) are lysyl oxidase–like proteins (LOXL)2, LOXL3 [81], and ILK [82]. A Snail 1especific phosphatase (Snail-1 activator) is C-terminal domain phosphatase [83]. Snail-2 is targeted for degradation by the direct action of p53 and the ubiquitin ligase Mdm2 [84].

In addition to protein translation and stability, the function of Snail-1 depends on nuclear localization mediated by Snail-1's nuclear localization sequence. The phosphorylation of human Snail-1 by p21-activated kinase 1 promotes the nuclear localization of Snail-1 (and therefore Snail-1 activation) in breast cancer cells [85]. In zebrafish, LIV-1 promotes the translocation of Snail-1 into the nucleus [86]. Snail-1 also contains a nuclear export sequence (NES) that depends on the calreticulin nuclear export pathway [87]. This NES sequence is activated by the phosphorylation of the same lysine residues targeted by GSK-3β, which suggests a mechanism whereby phosphorylation of Snail-1 by GSK-3β results in the export of Snail-1 from the nucleus and subsequent degradation.

LEF/TCF activity is also regulated by other proteins. β-Catenin is required as a cofactor for LEF/TCF-mediated activation of transcription, and Lef-1 can also associate with cofactor Smads to activate the transcription of additional EMT genes [88]. In colon cancer cells, thymosin β4 stabilizes ILK activity [89].

EMT transcription factors such as Snail-1, Zeb1, and Lef-1 are regulated by a variety of mechanisms at both the transcriptional and posttranscriptional levels by noncoding RNA translation control, protein degradation, nuclear localization, and cofactors such as β-catenin.

Molecular control of the epithelial–mesenchymal transition

The initiation of an EMT or MET is a tightly regulated event during development and tissue repair because deregulation of cellular organization is disastrous to the organism. A variety of external and internal signaling mechanisms coordinate the complex events of the EMT, and these same signaling pathways are often disrupted or reactivated during disease. EMTs or METs can be induced by either diffusible signaling molecules or ECM components. We next discuss the role of signaling molecules and ECM in triggering an EMT, and then present a summary model for EMT induction.

Ligand-receptor signaling

During development, five main ligand-receptor signaling pathways are employed: TGF-β, Wnt, receptor tyrosine kinase (RTK), Notch, and Hedgehog. These pathways, among others, have a role in triggering EMTs. Although the activation of a single signaling pathway can be sufficient for an EMT, in most cases an EMT or MET is initiated by multiple signaling pathways acting in concert.

Growth factor-β pathway

The TGF-β superfamily includes the TGF-β, activin, and bone morphogenetic protein (BMP) families. These ligands operate through receptor serine/threonine kinases to activate a variety of signaling molecules including Smads, mitogen-activated protein kinase (MAPK), PI3K, and ILK. Most EMTs studied to date are induced in part, or solely, by TGF-β superfamily members [90]. During

embryonic heart development, TGF-β2 and TGF-β3 have sequential and necessary roles in activating the endocardium to invade the cardiac jelly and from the endocardial cushions [91]. In the avian neural crest, BMP4 induces *Snail-2* expression [92]. In the EMT that transforms epithelial tissue into metastatic cancer cells, TGF-β acts as a tumor suppressor during early stages of tumor development, but as a tumor/EMT inducer at later stages [90,93]. TGF-β signaling may combine with other signaling pathways to induce an EMT. For example, in cultured breast cancer cells, activated Ras and TGF-β induce an irreversible EMT [94], and in pig thyroid epithelial cells, TGF-β and epidermal growth factor (EGF) synergistically stimulate the EMT [95].

One outcome of TGF-β signaling is to change epithelial cell polarity immediately. In a TGF β−induced EMT of mammary epithelial cells, TGF-βRII directly phosphorylates the polarity protein, Par6, leading to the dissolution of tight junctions [34]. TGF-β signaling also regulates gene expression through the phosphorylation and activation of Smads. Smads are important cofactors in the stimulation of an EMT. For example, Smad3 is necessary for a TGF β−induced EMT in lens and kidney tissue in vivo [96]. The Smad3/4 also complex with Snail-1 and corepress the promoters of cell−cell adhesion molecules [97]. Furthermore, TGF-βRI directly binds to and activates PI3K [98], which in turn activates ILK and downstream pathways.

ILK is emerging as an important positive regulator of EMTs [99]. ILK interacts directly with growth factor receptors (TGF-β, Wnt, or RTK), integrins, the actin skeleton, PI3K, and focal adhesion complexes. ILK directly phosphorylates Akt and GSK-3β, and results in the subsequent activation of transcription factors such as AP-1, NF-κB, and Lef-1. Overexpression of ILK in cultured cells causes the suppression of GSK-3β activity [82], translocation of β-catenin to the nucleus, activation of Lef-1/β-catenin transcription factors, and the downregulation of E-cadherin [100]. Inhibition of ILK in cultured colon cancer cells leads to the stabilization of GSK-3β activity, decreased nuclear β-catenin localization, the suppression of *Lef-1* and *Snail-1* transcription, and reduced invasive behavior of colon cancer cells [101]. ILK activity also results in Lef 1−mediated transcriptional upregulation of MMPs [60]. Hence, ILK (inducible by TGF-β signaling) is capable of orchestrating most of the major events in an EMT, including the loss of cell−cell adhesion and invasion across the basal lamina.

Wnt pathway

Many EMTs or METs are also regulated by Wnt signaling. Wnts signal through seven-pass transmembrane proteins of the Frizzled family, which activates G-proteins and PI3K, inhibits GSK-3β, and promotes nuclear β-catenin signaling. For example, during zebrafish gastrulation, Wnt11 activates the GTPase Rab5c, which results in the endocytosis of E-cadherin [102]. Wnt6 signaling is sufficient for increased transcription of *Snail-2* in the avian neural crest [103]. Snail-1 expression increases Wnt signaling [104], which suggests a positive feedback loop.

One of the downstream signaling molecules activated by Wnt signaling is β-catenin. β-Catenin is a structural component of adherens junctions. Nuclear β-catenin is also a limiting factor for the activation of LEF/TCF transcription factors. β-Catenin is pivotal for regulating most EMTs. Interfering with nuclear β-catenin signaling blocks the ingression of sea urchin PMCs [105], and in β-catenin mouse knockouts, the primitive streak EMT does not occur and no mesoderm is formed [106]. β-Catenin is also necessary for the EMT that occurs during cardiac cushion development [107]. In breast cancer, β-catenin expression is highly correlated with metastasis and poor survival [108], and blocking β-catenin function in tumor cells inhibits invasion in vitro [109]. It is unclear whether β-catenin overexpression alone is sufficient for all EMTs. If β-catenin is misexpressed in cultured cells, it causes apoptosis [110]. However, the misexpression of a stabilized from of β-catenin in mouse epithelial cells in vivo results in metastatic skin tumors [111].

Signaling by receptor tyrosine kinase ligands

The RTK family of receptors and the growth factors that activate them also regulate EMTs or METs. Ligand binding promotes RTK dimerization and activation of the intracellular kinase domains by autophosphorylation of tyrosine residues. These phosphotyrosines act as docking sites for intracellular signaling molecules, which can activate signaling cascades such as Ras/MAPK, PI3K/Akt, JAK/STAT, or ILK. We will cite a few examples of RTK signaling in EMTs and METs.

Hepatocyte growth factor (HGF; also known as scatter factor) acts through the RTK c-met. HGF is important for the MET in the developing kidney [112]. HGF signaling is required for the EMT that produces myoblasts (limb muscle precursors) from somite tissue in the mouse [10]. In epithelial cells, HGF causes an EMT through MAPK and early growth response factor-1 signaling [113].

Fibroblast growth factor (FGF) signaling regulates mouse primitive streak formation [114]. FGF signaling also stimulates cell motility and activates MMPs [115,116].

EGF promotes the endocytosis of E-cadherin [117]. EGF can also increase Snail-1 activity via the inactivation

of GSK3-β [118], and EGF promotes increased *Twist* expression through a JAK/STAT3 pathway [119].

Insulin growth factor (IGF) signaling induces an EMT in breast cancer cell lines by activating Akt2 and suppressing Akt1 [120]. In prostate cancer cells, IGF-1 promotes Zeb-1 expression [121]. In fibroblast cells, constitutively activated IGF-IR increases NF-κB activity and Snail-1 levels [122]. In several cultured epithelial cell lines, IGFR1 is associated with the complex of E-cadherin and β-catenin, and the ligand IGF-II causes the redistribution of β-catenin from the membrane to the nucleus, activation of the transcription factor TCF-3, and a subsequent EMT [123].

Another RTK known for its role in EMTs is the ErbB2/HER-2/Neu receptor, whose ligand is heregulin/neuregulin. Overexpression of HER-2 occurs in 25% of human breast cancers, and the misexpression of HER-2 in mouse mammary tissue in vivo is sufficient to cause metastatic breast cancer [124]. Herceptin (antibody against the HER-2 receptor) treatment is effective in reducing the recurrence of HER 2−positive metastatic breast cancers. HER-2 signaling activates *Snail-1* expression in breast cancer through an unknown mechanism [47]. The RTK Axl is also required for breast cancer carcinoma invasiveness [125].

Vascular endothelial growth factor (VEGF) signaling promotes Snail-1 activity by suppressing GSK3-β [126] and results in increased levels of *Snail-1*, *Snail-2*, and *Twist* [127]. Snail-1 can also activate the expression of VEGF [128]. RTK signaling is important for many EMTs.

Notch pathway

The Notch signaling family also regulates EMTs. When the Notch receptor is activated by its ligand Delta, an intracellular portion of the Notch receptor ligand is cleaved and transported to the nucleus, where it regulates target genes. Notch1 is required for cardiac endothelial cells to undergo an EMT to make cardiac cushions, and the role of Notch may be to make cells competent to respond to TGF-β2 [129]. In the avian neural crest EMT, Notch signaling is required for the induction and/or maintenance of *BMP4* expression [130]. Similarly, Notch signaling is required for the TGF β−induced EMT of epithelial cell lines [131], and Notch promotes *Snail-2* expression in cardiac cushion cells [132] and cultured cells [133].

Hedgehog pathway

The hedgehog pathway is also involved in EMTs. Metastatic prostate cancer cells express high levels of hedgehog and *Snail-1*. If prostate cancer cell lines are treated with the hedgehog-pathway inhibitor, cyclopamine, levels of *Snail-1* are decreased. If the hedgehog-activated transcription factor, Gli, is misexpressed, *Snail-1* expression increases [134].

Additional signaling pathways

Other signaling pathways that activate EMTs include inflammatory signaling molecules, lipid hormones, ROS species, and hypoxia. Interleukin-6 (inflammatory and immune response) can promote Snail-1 expression in breast cancer cells [135], and Snail-1, in turn, can activate interleukin-6 expression [136], providing a link between inflammation and EMTs [137]. The lipid hormone prostaglandin E2 (PGE2) induces Zeb1 and Snail activity in lung cancer cells [138], and Snail-1 can also induce PGE2 expression [139]. ROS species can also activate EMTs by PKC and MAPK signaling [140]. Hypoxia is important for initiating EMTs during development [141] and disease [137], often through hypoxia-inducible factor-1, which directly activates *Twist* expression [142]. Hypoxia also activates lysyl oxidases, which stabilize Snail-1 expression [143] by inhibiting GSK-3β activity [144].

In addition to diffusible signaling molecules, ECM molecules regulate EMTs or METs. This was first dramatically demonstrated when lens or thyroid epithelium was embedded in collagen gels, and then promptly underwent an EMT [2]. Integrin signaling appears to be important in this process [145] and involves ILK-mediated activation of NF-κB, Snail-1, and Lef-1 [146]. Other ECM components that regulate EMTs include hyaluronan [147], the γ-2 chain of laminin 5 [148], periostin [149], and podoplanin [150,151]. Avariety of diffusible signals and ECM components can stimulate EMTs or METs.

A model for epithelial−mesenchymal transition induction

Many experimental studies on EMT mechanisms are piecework, and although great progress has been made in discovering EMT pathways, the entire signaling network is still incomplete. Fig. 5.2 shows many of the various signaling mechanisms, although in actuality only a few of the inductive pathways will be used for individual EMTs. From experimental evidence, it appears that many EMT signaling pathways converge on ILK, the inhibition of GSK-3β, and stimulation of nuclear β-catenin signaling to activate Snail and LEF/TCF transcription factors. Snail, Zeb, and LEF/TCF transcription factors then act on a variety of targets to suppress cell−cell adhesion, induce changes in cell polarity, stimulate cell motility, and promote invasion of the basal lamina.

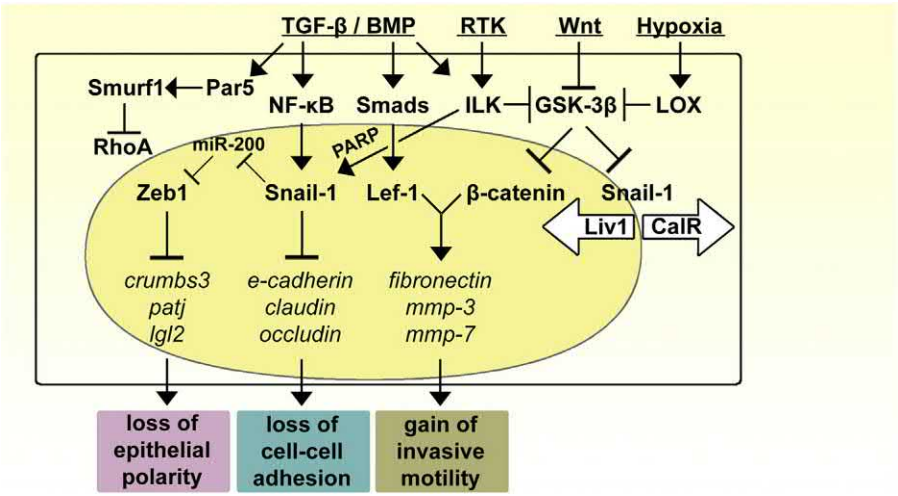

FIGURE 5.2 **Induction of an EMT.*EMT*, epithelial−mesenchymal transition;** This figure summarizes some of the important molecular pathways that bring about an EMT. Many of the signaling pathways converge on the activation of Snail-1 and nuclear β-catenin signaling to change gene expression, which results in the loss of epithelial cell polarity, the loss of cell−cell adhesion, and increased invasive cell motility. *BMP*, bone morphogenetic protein; *CalR*, calreticulin; *GSK-3β*, glycogen synthase kinase 3β; *Igl2*, immunoglobulin 2; *ILK*, integrin-linked kinase; *LOX*, lysyl oxidase; *miR-200*, microRNA-200; *mmp*, matrix-metalloprotease; *NF-κB*, nuclear factor κB; *RhoA*, Ras homolog gene family, member A; *RTK*, receptor tyrosine kinase; *TGF-β*, transforming growth factor-β; *Zeb1*, zinc finger E-box-binding homeobox 1.

Conclusion

Since the term "EMT" was coined [10], important insights have been made in this rapidly expanding field of research. EMT and MET events occur during development, tissue repair, and disease, and many molecules that regulate the various EMTs or METs have been characterized, thanks in large part to the advent of cell culture models. However, the EMT regulatory network as a whole is still incomplete. An improved understanding of EMT and MET pathways will lead to more effective strategies for tissue engineering and novel therapeutic targets for the treatment of disease.

List of acronyms and abbreviations

BMP	bone morphogenetic protein
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial−mesenchymal transition
FGF	fibroblast growth factor
GSK-3β	glycogen synthase kinase 3β
HGF	hepatocyte growth factor
IGF	insulin growth factor
ILK	integrin-linked kinase
LEF/TCF	lymphoid enhancer-binding factor/T-cell factor
LOXL proteins	lysyl oxidase−like proteins
MDCK cells	Madin−Darby canine kidney cells
MET	mesenchymal-epithelial transition
MMPs	matrix-metalloproteases
MRTFs	myocardin-related transcription factors
NES	nuclear export sequence
PGE2	prostaglandin E2
PI3K	phosphatidylinositol 3 kinase
PMC	primary mesenchyme cells
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor
Zeb	zinc finger E-box-binding

Glossary

Apical	surface of the epithelial layer where adherens junctions and tight junctions are located. This is opposite the basal surface
Basal	surface of the epithelial layer where the basal lamina is found. This is opposite the apical surface
Basal lamina	consists of extracellular matrix components such as collagen type IV, fibronectin, and laminin. The basal lamina functions to stabilize the epithelium and is a barrier against migratory cells
Epithelial	epithelial cells adhere tightly to each other at their lateral surfaces and to an organized extracellular matrix at their basal domain, thereby producing a sheet of cells resting on a basal lamina with an apical surface

Epithelial–mesenchymal transition	The conversion of epithelial cells into mesenchymal cells
Mesenchymal	mesenchymal cells are individual cells with a bipolar morphology that are held together as a tissue within a three-dimensional extracellular matrix
Mesenchymal–epithelial transition	The conversion of mesenchymal cells into epithelial cells

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Chapter 6

The dynamics of cell–extracellular matrix interactions, with implications for tissue engineering

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Introduction

Historical background

For many years the extracellular matrix (ECM) was thought to serve only as a structural support for tissues. This began to change as multiple research groups observed differences in cell structure and organization, as well as changes in gene expression, when cells were cultured in the presence or absence of ECM components. For example, mammary gland epithelial cells cultured on Engelbreth–Holm–Swarm (EHS), a specialized matrix synthesized by EHS tumors with many of the properties of basement membranes, synthesized milk molecules and underwent the branching morphogenesis characteristic of mammary glands [1]. These and other findings implicating matrix molecules in myotube formation and morphogenesis of multiple tissues and organs suggested that cells express receptors for matrix molecules that, when bound to their respective matrix molecule(s), are critical for embryonic development [2]. This led to the development of the model called dynamic reciprocity, in which ECM molecules interact with specific cell-surface receptors and initiate intracellular signal transduction cascades, promoting expression of specific genes whose products, in turn, affect the ECM [2]. This set the stage for further investigations into the mechanisms by which ECM molecules influence cell behavior. These mechanisms regulated by matrix molecules include changes in cell adhesion, migration, growth, differentiation, and apoptosis, modulation of cytokine and growth factor signaling, and stimulation of intracellular signaling.

Evidence for the mechanisms underlying the roles of cell–matrix interactions in the regulation of cell signaling events and cell behaviors come from a combination of in vitro cell cultures with matrix molecules and in vivo experiments in animals lacking specific matrix molecules or their receptors. After describing some of the most prominent ECM molecules and their receptors later, we discuss the importance of selected cell–matrix interactions and their associated signal transduction in development and wound healing. We then end with a discussion of the relevance of cell–matrix interactions in designing engineered tissues.

Extracellular matrix composition

The extracellular microenvironment contains both traditional structural matrix components, such as fibronectin, hyaluronic acid, proteoglycans, collagens, glycosaminoglycans, and elastins, and nonstructural matricellular proteins, including secreted protein acidic and rich in cysteine (SPARC), tenascin, osteopontin, and thrombospondins. The distribution and organization of these molecules is not static, but varies from tissue to tissue and, during development, from stage to stage, which has significant implications for tissue functions [3]. For example, mesenchymal cells are immersed in an interstitial matrix that confers specific biomechanical and functional properties to connective tissue, whereas epithelial and endothelial cells contact a specialized matrix, the basement membrane, via their basal surfaces only, conferring mechanical strength and specific physiological properties to the epithelia [4]. Alterations in the temporal and spatial composition, organization, and

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distribution of ECM during development and tissue repair results not only from differential gene expression of certain matrix proteins in specific tissues, but also from changes in their alternative splicing and posttranslational modifications, as alternative splicing may change their binding potential to other matrix molecules and/or receptors. Furthermore, variations in glycosylation can alter cell adhesion and migration, and proteolytic cleavage can generate fragments with diverse biological functions [5–7].

In addition to the direct effects exerted by matrix molecules on tissue development and function, the ECM indirectly affects tissue function through its interactions with nonmatrix proteins such as growth factors and cytokines. Therefore tissue-specific and developmental stage–specific variations in ECM composition may thereby regulate the ability of these associated molecules to initiate intracellular signaling and influence cell behavior by limiting their diffusion, protecting them from degradation, presenting them more efficiently to their receptors, and/or sequestering them away from their receptors [3,8]. For example, while vascular endothelial growth factor (VEGF) binding to heparan sulfate proteoglycans (HSPGs) enhances its binding to and activation of VEGF receptors, proteoglycan binding to heparin-binding epidermal growth factor (EGF) (HB-EGF) interferes with the activation of EGF receptors HB-EGF until its release by proteoglycan degradation [9,10]. Beyond proteoglycan facilitation of growth factor and cytokine signaling, specific domains of some ECM molecules or fragments derived from their proteolysis, referred to as matricryptins, can directly bind growth factor receptors and influence their downstream signaling. Either within the context of intact matrix molecules or following protease-mediated matricryptin release, the EGF-like repeats of laminin or tenascin-C can bind and activate the EGF receptor (EGFR), while the proteoglycan decorin inhibits several growth factor receptors, including EGFR and VEGFR2 [8,11]. Regulation of growth factor receptors by intact ECM molecules may facilitate a stable signaling environment for the associated cells because the ligands are unable to diffuse or be internalized, creating persistent signaling through the growth factor receptors [12,13]. In contrast, following proteolytic cleavage of matrix components, released matricryptins that bind and inhibit VEGFR2, such as endorepellin and endostatin, are more likely to diffuse away from their generation sites and can be internalized, suggesting more transient impacts on cell function [5,8,10]. Taken together, matrix molecules, growth factors, and growth factor receptors form a complex network of interactions, with similarly complex effects on cell survival and function.

Receptors for extracellular matrix molecules

Many cell-surface receptors mediate cell–matrix interactions that connect extracellular binding events to intracellular signaling pathways. Integrins, a family of heterodimeric transmembrane proteins composed of α and β subunits, were the first ECM receptors to be identified. The 18 α and 8 β subunits pair in various combinations to yield 24 separate heterodimers, many of which recognize specific sequences on their respective ligands, including the well-characterized RGD motif present in fibronectin, vitronectin, thrombospondin, and fibrinogen (Fig. 6.1; [14]). Some integrin heterodimers exhibit a high degree of ligand specificity, while others interact with multiple possible ligands (Fig. 6.1), facilitating plasticity and redundancy in specific systems [14]. Apart from integrin β_4, the integrins have large extracellular domains and very small intracellular domains. Despite the small size of their cytoplasmic domains, integrins bind a variety of intracellular proteins, facilitating their intracellular connections with the cytoskeleton and activation of signal transduction pathways [15,16]. Complicating our understanding of integrin-mediated, matrix-induced signaling is the ability of multiple integrins to directly bind nonmatrix molecules, including the adhesion molecules ICAM1-3, VCAM1, and RGD-containing cadherins, thereby mediating cell–cell adhesions, and the interactions and coordinated signaling of fibroblast growth factor (FGF) receptor (FGFR) and VEGFR2 with integrin $\alpha_v\beta_3$ [17,18]. Despite this complexity, integrin knockout and inhibition experiments have elucidated many of the matrix-induced integrin signaling events described later in more detail.

In addition to integrins, several transmembrane proteoglycans, including syndecans, RHAMM (receptor for hyaluronan-mediated motility), and CD44, can also serve as receptors for ECM molecules, including collagen, fibronectin, laminin, and hyaluronan [7]. Syndecans 1–4 mediate cell–ECM interactions via chondroitin- and heparan-sulfate glycosaminoglycans, whose composition varies based upon the type of syndecan and the tissue in which it is expressed. These differential glycosaminoglycan modifications alter the binding capacity of ligands such as fibronectin and tenascin [19]. The short cytoplasmic domains of syndecans can interact with signaling proteins and the cytoskeleton and thereby induce signal transduction upon binding to their ECM ligands, resulting in changes in cell adhesion and migration [20]. Although syndecan activation by its matrix ligands can directly activate downstream signaling, syndecan also mediates the formation of larger signaling complexes that activate signaling in an indirect manner. In these complexes the protein core of syndecan directly binds some integrins, while the heparan sulfate moieties of syndecan bind several growth factors and matrix molecules, ultimately facilitating ligand-mediated activation of growth factor receptors and integrins [20].

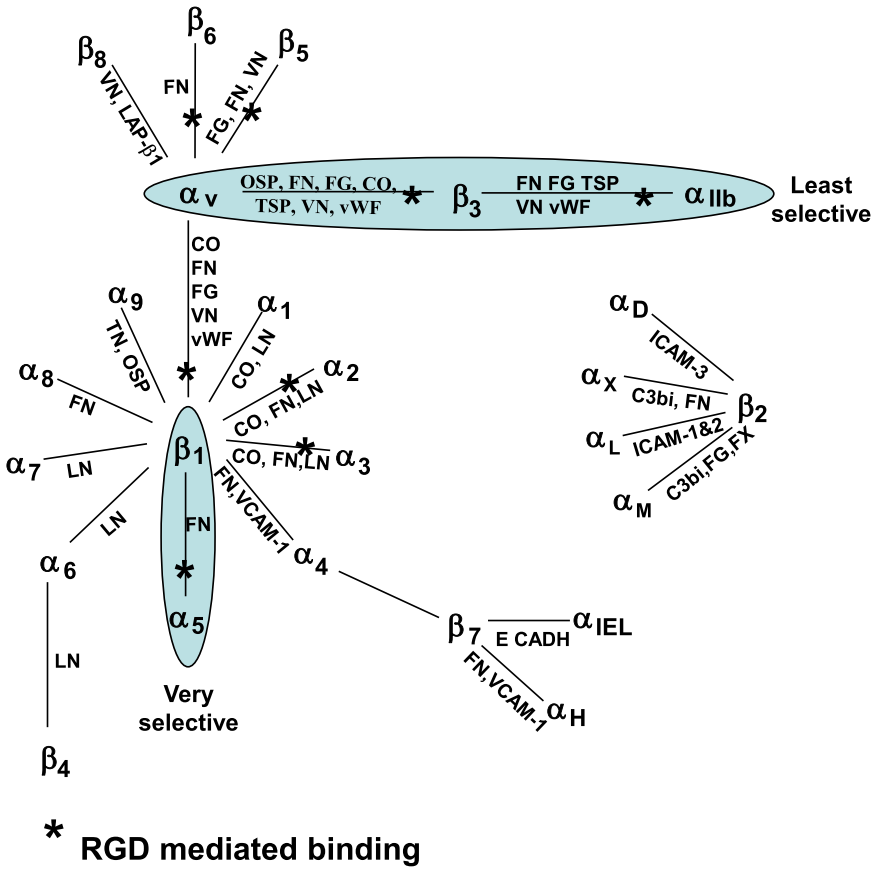

FIGURE 6.1 Members of the integrin family of ECM receptors and their respective ligands. These heterodimeric receptors are composed of one a and one b subunit, and are capable of binding a variety of ligands, including Ig superfamily cell adhesion molecules, complement factors, and clotting factors in addition to ECM molecules. Cell–cell adhesion is largely mediated through integrin heterodimers containing the b_2 subunits, while cell–matrix adhesion is mediated primarily via integrin heterodimers containing the b_1 and b_3 subunits. In general, the b_1 integrins interact with ligands found in the connective tissue matrix, including laminin, fibronectin, and collagen, whereas the b_3 integrins interact with vascular ligands, including thrombospondin, vitronectin, fibrinogen, and von Willebrand factor. *CO*, Collagens; *C3bi*, complement component; *ECM*, extracellular matrix; *FG*, fibrinogen; *FN*, fibronectin; *FX*, Factor X; *ICAM-1*, intercellular adhesion molecule-1; *ICAM-2*, intercellular adhesion molecule-2; *ICAM-3*, intercellular adhesion molecule-3; *LN*, laminin; *OSP*, osteopontin; *TN*, tenascin; *TSP*, thrombospondin; *VCAM-1*, vascular cell adhesion molecule-1; *VN*, vitronectin; *vWF*, von Willebrand factor.

Similar to syndecans, the proteoglycan receptor CD44 interacts with multiple matrix ligands, including collagen IV, collagen XIV, fibronectin, osteopontin, and laminin, in addition to its primary ligand, hyaluronan [21]. Binding to these ligands is regulated, at least in part, by the tissue-specific splicing and glycosylation that yield multiple CD44 isoforms [22]. CD44 can also interact with another hyaluronan receptor, RHAMM, a peripheral membrane receptor that must bind transmembrane protein(s) like CD44, integrins, and/or receptor tyrosine kinases in order to transmit the signal from hyaluronan to intracellular signaling proteins [23]. Hyaluronan is present in most tissues in a high molecular weight, native form, but hyaluronidases and reactive oxygen or nitrogen species can generate lower molecular weight fragments following tissue injury and during tissue inflammation and repair [24]. The native and cleaved forms of hyaluronan elicit different cellular responses, likely due to differential receptor selectivity. CD44 binds more stably to high molecular weight hyaluronan than low molecular weight hyaluronan fragments, whereas hyaluronan fragments, but not the native high molecular weight form, bind and activate toll-like receptors (TLRs), strongly suggesting that the fragments function as "danger signals" that sense tissue damage and induce inflammatory responses [21,23].

Beyond proteoglycans and integrins, the elastin–receptor complex (ERC), TLRs, CD36, and receptor tyrosine kinases can also serve as matrix receptors. The ERC is a complex of three proteins: neuraminidase 1, cathepsin A, and the elastin-binding protein (EBP). EBP binds specifically to VGVAPG-repeating peptides and the GxxPG sequence found in elastin, fibrillin, laminin, and fragments derived from these matrix molecules, and this EBP binding initiates signal transduction through the neuraminidase-1

that is important in both mechanotransduction and elastin deposition [25,26]. Following tissue injury, active proteases generate elastin-derived peptides that then promote proliferation and/or migration of fibroblasts, epithelial/endothelial cells, and monocytes downstream of the ERC [26]. These effects suggest that elastin-derived peptides are able to promote wound healing, an idea supported by evidence that these peptides enhance the healing of burn wounds when used in conjunction with more conventional treatments [27].

In addition to the ERC, TLRs and CD36 can function as matrix receptors and thereby affect cell function. Fibronectin and low molecular weight fragments of hyaluronan bind and activate TLRs that are also activated by microbial molecules, suggesting that fragments of matrix molecules may function as "danger signals" that induce inflammation following tissue damage and the matrix degradation that accompanies the healing process [5,23]. The scavenger receptor CD36 interacts with thrombospondin, collagen I, and collagen V, and, in endothelial cells, thrombospondin binding to CD36 induces apoptosis and is thus antiangiogenic in vivo [28,29]. Interestingly, both CD36 and TLR 4 interact with and are modified by the neuraminiase-1 subunit of the ERC, suggesting that ERC-binding elastin-derived peptides can regulate the course of wound inflammation and angiogenesis by altering the ligand binding and/or signaling downstream of these receptors [30].

Receptor tyrosine kinases can also function as matrix receptors. The receptor tyrosine kinase category of matrix receptors includes EGFR and VEGFR2, which interact with matrix molecules and proteolytically released matricryptins as described before, and the discoidin domain receptors DDR1 and DDR2 are activated by fibrillar collagens [31,32]. Unlike most other receptor tyrosine kinases, which are activated by dimerization, DDR1 and DDR2 exist as constitutive homodimers, suggesting an alternative mechanism of activation. Upon ligand binding, the discoid domain receptors (DDRs) undergo autophosphorylation and induce multiple downstream signaling pathways that remain active over long time periods, likely reflecting the long-lived nature of their collagen ligands [33]. DDR2 participates in the fibroblast recruitment and myofibroblast differentiation critical for wound healing, as both processes are reduced during wound healing in DDR2-deficient mice [33]. This function of DDR2, coupled with its prolonged signaling following ligand binding, suggests that collagen-DDR signaling may provide a stable signal for fibroblast recruitment and myofibroblast proliferation throughout the healing process.

Next, we will first discuss selected examples that illustrate the dynamics of cell–ECM interactions during development and wound healing, as well as the potential mechanisms involved in the signal transduction pathways initiated by these interactions. At the end, we will discuss the implications of cell–ECM interactions in tissue engineering.

Cell–extracellular matrix interactions

Multiple biological processes, including those relevant to development and wound healing, require interactions between cells and their environment and the regulated modulation of such interactions. During development, the cellular cross talk with the surrounding ECM promotes the formation of patterns, the development of form (morphogenesis), and the acquisition and maintenance of differentiated phenotypes during embryogenesis. Similarly, during wound healing these interactions contribute to the processes of clot formation, inflammation, granulation tissue development, and remodeling. As outlined next, the current body of research in the fields of both embryogenesis and wound healing implicates multiple cellular behaviors, including cell adhesion/deadhesion, migration, proliferation, differentiation, and apoptosis (programed cell death), in these critical events.

Development

Adhesion and migration

Today, there is a vast body of experimental evidence that demonstrates the direct participation of ECM in cell adhesion and migration, but some of the most compelling experiments came from early studies in gastrulation, migration of neural crest cells (NCCs), angiogenesis, and epithelial organ formation. Cell interactions with fibronectin are important during gastrulation. Inhibition of fibronectin–integrin interactions in amphibian embryos by the introduction of blocking antibodies or RGD-containing peptides, which compete with integrins for ECM binding, into their blastocoel cavities disrupts normal cell movement and leads to abnormal development, while introduction of recombinant fibronectin lacking the RGD motif perturbs amphibian gastrulation [34,35]. These effects are not unique to fibronectin, as they can also be introduced by manipulation of other molecules, such as hyaluronan and HSPGs. Inhibition of hyaluronan synthesis in zebra fish embryos interferes with cell movements in gastrulation, potentially due to a defect in Rac1 activation, as expression of constitutively active Rac rescued the observed migratory defects [36]. HSPGs are also critical for gastrulation, likely resulting from their regulation of fibronectin and laminin expression as well as their ability to regulate growth factor signaling. A mutation of glypican-4, a HSPG, both increases fibronectin and laminin expression and inhibits polarized cell migration during somite formation, likely due to Rac1 redistribution across the entire membrane rather than

localization at a leading edge [37]. This change in Rac1 localization may result from the change in fibronectin or laminin signaling through integrins, a change in HSPG regulation of growth factor signaling, or both. Binding of fibronectin to HSPG causes a conformational change in fibronectin, exposing growth factor binding sites that then bind platelet-derived growth factor (PDGF)-AA, generating a stable PDGF gradient that promotes the directional cell migration critical for gastrulation [38]. HSPGs are also necessary for FGF signaling during gastrulation, as inhibition of HSPG synthesis alters FGF localization, inhibits FGF signaling, and arrests mouse embryonic development at gastrulation [39].

In addition to their roles in gastrulation, cell−matrix interactions are also important for the migration of NCCs, which develop in the dorsal portion of the neural tube just after closure of the tube, deadhering from each other and then migrating extensively throughout the embryo in ECM-filled spaces, giving rise to a variety of phenotypes. The importance of cell−ECM interactions in NCC migration is supported by studies performed in the white mutant of Mexican axolotl embryos. The NCC that give rise to pigment cells fail to emigrate from the neural tube in these embryos, but when microcarriers containing subepidermal ECM from normal embryos are implanted into the appropriate area in these mutants, the NCC pigment cell precursors emigrate normally [40]. Laminin and fibronectin, the latter of which appears between chick NCC just prior to their emigration from the neural tube, play particularly critical roles in this process [41]. Inhibition of fibronectin, laminin-111 (laminin-1), laminin-411 (laminin-8), or their integrin receptors, using function-blocking antibodies, competing peptides, or antisense RNA, prevents NCC migration, while exogenous laminin or fibronectin is sufficient to induce premature NCC migration [40,42]. More recent studies have identified different subsets of NCC, which may exhibit different responses to specific matrix molecules. For example, cranial NCC does not migrate in response to fibronectin but do migrate on laminin [43]. Matrix remodeling also contributes to NCC migration and the initial epidermal-to-mesenchymal transition that allows NCC deadhesion from its site of origin, as several matrix metalloproteinases (MMPs) are required for deadhesion and/or migration events. MMP-9, for example, is necessary for NCC deadhesion and migration during avian development. The role of MMP-9 in deadhesion may be related to its ability to cleave the cell−cell adhesion molecule N-cadherin, while its role in migration may involve laminin degradation [44]. Proteoglycans also participate in the NCC migration process. Aggrecan and versican, proteoglycans that predominate in tissues surrounding the migrating NCC, inhibit NCC migration and are thought to form nonadhesive boundaries that delimit NCC migration pathways [40,45]. In contrast, syndecan-4 facilitates directional migration of NCC. In conjunction with a noncanonical Wnt pathway, syndecan-4 inhibits Rac1 at the rear of the cell, restricting Rac activity to the leading edge of the migrating cell and facilitating directional migration [46].

Cell−matrix interactions are also critical for the endothelial cell adhesion and migration events necessary for angiogenesis, a process in which new blood vessels form from preexisting vessels. Early indications of the role of ECM in angiogenesis were observed when human umbilical vein endothelial cells (HUVEC) were cultured on EHS tumor matrix (Matrigel) that resembles the composition of basement membranes, with large amounts of laminin, collagen IV, entactin/nidogen, and proteoglycans. When HUVEC are cultured on Matrigel for 12 hours, they migrate and form tube-like structures. In contrast, when these cells are cultured with collagen I, they only form tube-like structures after they are maintained *inside* the gels for one week, at which time the cells have secreted their own basement membrane molecules [47]. The observation that tube formation occurs more rapidly on Matrigel than within collagen gels strongly suggested an important role for one or more of the matrix molecules present within the basement membrane in the development of capillary-like endothelial tubes. Indeed, laminin-111, the predominant matrix molecule in Matrigel, induces endothelial cell adhesion, migration, and tube formation in vitro, as well as angiogenesis in vivo [48]. Similarly, overexpression of laminin-411, the predominant laminin isoform in vascular basement membranes, increases endothelial cell migration in an integrin α_6- and β_1-dependent manner, while mice lacking expression of laminin α_4 exhibit defects in capillary basement membrane formation, suggesting that laminin-411 participates in angiogenesis and vascular maturation, respectively [49,50]. Antibody-mediated inhibition and shRNA-mediated knockdown of integrin α_6, the α subunit of laminin receptors $\alpha_6\,\beta_1$ and $\alpha_6\,\beta_4$, reduced endothelial tube formation and endothelial cell migration on and adhesion to basement membrane extracts, and decreased FGF-induced angiogenesis in the chick chorioallantoic membrane (CAM) assay [51]. Taken together, these data implicate laminin/integrin interactions in blood vessel development and stabilization.

Along with laminin, several other matrix molecules are critical for developmental blood vessel formation and maturation, including collagen IV, the HSPG perlecan, and nidogen [50]. Mice deficient in any of these three proteins have defective basement membrane formation, and collagen IV is also critical in the ability of the vasculature to withstand mechanical stress [50]. Nidogen and perlecan each interact simultaneously with collagen IV and laminins to organize and stabilize the basement membrane, while perlecan, an HSPG, can bind proangiogenic

growth factors and promote receptor binding, thereby facilitating angiogenesis induced by these factors [50]. VEGF and PDGF interaction with HSPG can sequester these factors until their release by proteases, limit their diffusion, and/or function as coreceptors to promote receptor binding and activation [50]. Binding of VEGF to HSPG appears to be important in its localization and function during development. In mouse embryos, sole expression of a VEGF isoform that lacks the heparin-binding domain, VEGF121, increases diffusion of VEGF from the site of secretion and decreases blood vessel branching. This altered vascular patterning appears to result from impaired endothelial cell migration, as shown by decreased filopodia formation in the migrating "tip cell" of nascent sprouts [52]. In embryos solely expressing the HSPG-binding VEGF isoform, VEGF188, branching of the blood vessels and filopodia formation by endothelial tip cells were increased when compared with wild type [52]. HSPG are also important for vessel stability and maturation, as HSPG are required for PDGF-BB-induced signaling, which, in turn, is important for pericyte migration and interaction with nascent blood vessels [50].

In contrast to the proangiogenic activities of many matrix molecules, matricryptins released by their proteolysis can exert antiangiogenic effects. Many soluble integrin ligands released through proteolytic activity are antiangiogenic, such as tumstatin and arresten (derived from collagen IV), endostatin (derived from collagen XVIII), and endorepellin (derived from perlecan) [5]. These matricryptins could inhibit angiogenesis by interacting with different receptors, activating alternative signaling pathways downstream of the same receptors, and/or compete with the intact matrix molecules for receptor binding. In the last case the displacement of the intact matrix molecules by soluble matricryptins would alter the presentation of the ligands to their receptors, resulting in changes in mechanical resistance that alter signaling events downstream of the receptor and yielding different cellular responses [53]. Although the ways in which matricryptins and intact matrix molecules induce different cellular outcomes are not completely understood, the fact that soluble and intact ECM receptor ligands may, at times, lead to alternative outcomes is likely of importance in vivo following matrix degradation. During angiogenesis, endothelial cell migration and invasion into surrounding tissues are accompanied by the activation of matrix-degrading enzymes, which then cleave the matrix and release both matrix-bound growth factors and generate ECM fragments, providing additional angiogenic or antiangiogenic cues to further influence the process [3]. Therefore matrix molecules that initially facilitate angiogenesis may be proteolytically cleaved to create antiangiogenic matrix fragments, preventing additional blood vessel formation and/or resulting in vessel maturation. Thus the temporal and spatial production and cleavage of matrix molecules may have important consequences for tissue homeostasis. However, the contribution of matricryptins to developmental angiogenesis and vessel maturation remains unclear due to difficulty in distinguishing between cleaved and uncleaved matrix molecules in tissue sections and an inability to delete the matricryptin without also mutating the parent matrix molecule.

Proliferation

Many cell–ECM interactions directly modulate cell proliferation, with some matrix molecules inducing and others inhibiting proliferation. Fibronectin and tenascin-C stimulate cell proliferation in vitro via their EGF-like repeats, which bind and activate the EGFR [8,54,55]. In contrast, laminin inhibits cell proliferation in vitro. Normal human breast cells cultured on plastic continue to divide but stop dividing if grown in a basement membrane matrix, likely due to the laminin-111 present in this matrix mixture [56,57]. Similarly, laminin inhibits endothelial cell proliferation through integrin $\alpha_3\beta_1$, providing a potential mechanism whereby the vascular basement membrane maintains vessel quiescence in the absence of angiogenic stimuli [58].

Some of the ECM effects on cell proliferation are indirect, requiring matrix cooperation with growth factors. As mentioned before under migration/adhesion, binding of growth factors to matrix molecules can affect their interactions with their receptors, limit their diffusion, and/or sequester them until protease-mediated release. Several matrix-associated growth factors, including HB-EGF and TGFβ, can regulate cell proliferation during development.

The importance of HB-EGF in cell proliferation during development is exemplified by its role in the proliferation of vascular smooth muscle cells (VSMCs). Mice expressing Heparin Sulphate (HS)-deficient perlecan exhibit increased VSMC proliferation in vivo following vascular injury and enhanced growth factor-induced VSMC proliferation in vitro [59]. This suggests that the perlecan core protein promotes VSMC proliferation and/or that the HS-modified perlecan inhibits VSMC proliferation. While there is some evidence for the former, many more studies have elucidated the ability of heparan sulfate on HSPG to bind growth factors and regulate their receptor binding and downstream signaling. Expression of perlecan without the growth factor–binding HS moieties leaves proproliferative growth factors free to interact with their receptors and induce proliferation. One such growth factor is HB-EGF, an HSPG-binding growth factor whose protease-mediated release from HSPG enables receptor activation and, in VSMC, ultimately induces proliferation [9]. A mouse embryo expressing a mutant

HB-EGF that could not be cleaved and released from HSPG showed defects resembling the HB-EGF knockout, with abnormal heart development and spontaneous fibrosis, suggesting that HB-EGF release is necessary for its function [60]. In contrast, embryos expressing a soluble version of HB-EGF that could not bind HSPG showed excessive proliferation of keratinocytes, suggesting the importance of HSPG binding in the regulation of HB-EGF-induced keratinocyte proliferation during development [60].

As with proteoglycan binding to HB-EGF, binding of the proteoglycans biglycan and decorin to TGFβ inhibits its receptor binding and downstream signaling until proteolytic cleavage of these proteoglycans releases TGFβ [61]. The proteoglycan betaglycan forms a complex with TGFβ and its receptor to effectively present the ligand to the receptor and induce signaling [62]. Because TGFβ-induced signaling in VSMC can either induce or repress their proliferation, dependent upon the concentration of TGFβ present, the relative abundance of decorin, biglycan, and betaglycan may be critical in determining the amount of TGFβ that can activate its receptors, which may then determine whether the VSMC proliferate in response to TGFβ [63].

TGFβ-binding proteoglycans may also regulate its signaling during early stages of mammary gland development occurring in puberty [64]. During this period, inductive events mediated by the basement membrane take place between the epithelium and the surrounding mesenchyme, which play an important role in epithelial proliferation during gland branching. Endogenous TGFβ produced by the ductal epithelium and surrounding mesenchyme forms complexes with mature periductal ECM but is absent from the nascent ECM in proliferating regions of newly forming ducts [65]. This TGFβ, after its activation from a latent to an active form, may participate in stabilizing the epithelium by stimulating expression of matrix molecules, inhibiting cell proliferation, and/or inhibiting matrix-degrading enzymes. In support of this possibility the mammary gland cells in transgenic mice expressing a kinase-deficient TGFβ receptor showed excessive proliferation, whereas application of exogeneous TGFβ or TGFβ overexpression in mammary epithelial cells decreased cell proliferation [64]. Although it is unclear how the interactions between TGFβ and proteoglycans can influence its function in this context, mice whose mammary epithelial cells are deficient in Ext1, an enzyme needed to synthesize heparan sulfate, undergo reduced mammary gland branching and duct formation, suggesting that HSPG do participate in this process [66]. It remains to be seen whether the heparan sulfate–associated betaglycan, which binds TGFβ and facilitates its downstream signaling, participates in TGFβ-repressed proliferation.

Differentiation

Matrix molecules are critical in regulating the differentiation of multiple cell types during development. Here, we focus on two types of developmental differentiation: mammary gland epithelial cells and keratinocytes.

In the mouse mammary gland the basement membrane and its individual components, in conjunction with lactogenic hormones, are responsible for the induction of the differentiated phenotype of the epithelial cells. When mid-pregnant mammary epithelial cells are cultured on plastic, they do not express mammary-specific genes. However, when the same cells are plated and maintained on basement membrane components (Matrigel), they form alveolar-like structures and exhibit the fully differentiated phenotype with expression of genes encoding milk proteins, such as β-casein [1]. Function-blocking antibodies and conditional knockouts identified laminin-111 as the ECM molecule present in Matrigel ultimately responsible for the observed differentiation, and that integrins β_1 and α_6 are critical in maintaining the differentiated state [1]. One role of laminin-111 in this process is in establishing mammary epithelial cell polarity, which redistributes the prolactin receptor to the apical surface of the epithelium and facilitates ligand binding, receptor activation, and signaling needed to induce expression of β-casein [67]. ECM molecules also regulate expression of another milk protein, the whey acidic protein (WAP). Matrigel decreases the production of TGFα by mammary gland epithelial cells, thereby increasing expression of WAP, which is otherwise inhibited by TGFα [68]. WAP may then participate in the maintenance of mammary epithelial differentiation by inhibiting the activity of laminin-degrading enzymes [69].

Like mammary epithelial cells, keratinocytes are epithelial cells that terminally differentiate. Keratinocytes form the stratified epidermal layers of the skin, in which the basal layer is highly proliferative, does not express the markers for terminal differentiation, and is the only cell layer in contact with the basement membrane. As these cells divide, the daughter cells lose contact with the basement membrane, move up to the suprabasal layers, and begin to express differentiation markers [70]. This suggests that physical interaction with the basement membrane may repress basal keratinocyte differentiation. Indeed, the basement membrane component fibronectin inhibits differentiation of the basal cells, as does activation of β1 integrin, while the keratinocytes in a conditional integrin β1 skin knockout mouse exhibit reduced proliferation and increased differentiation [70,71]. This suggests that fibronectin–integrin interactions prevent keratinocyte terminal differentiation in the basal keratinocyte layer, which is important in maintaining the proliferative capacity of these cells. Other basement membrane

components, including collagen IV and laminin-511, facilitate keratinocyte proliferation in culture, suggesting that these molecules may promote proliferation of the basal keratinocyte layer in vivo [72,73]. As such, multiple basement membrane components may work together to regulate proliferation of these cells by promoting cell proliferation and by preventing terminal differentiation.

Apoptosis

Programed cell death occurs during embryogenesis of higher vertebrates in areas undergoing remodeling, as in the development of the digits, palate, nervous system, in the selection of thymocytes in the thymus, during mammary gland involution, and during angiogenesis. In the mammary gland, for example, intact basement membrane molecules promote proliferation, differentiation, and survival of mammary epithelial cells, whereas matrix fragments induce apoptosis during the involution of the mammary gland [74]. The numerous alveoli that produce milk during lactation regress and are resorbed during involution due to enzymatic degradation of alveolar basement membrane and apoptosis [64,75]. During this involution, apoptosis appears to proceed in two distinct phases, an early phase characterized by increased levels of proinflammatory and apoptosis-associated proteins, including several members of the tumor necrosis factor (TNF) and TNF receptor superfamilies as well as the proapoptotic Bcl family member Bax [75]. This is then followed by a later apoptotic phase in which cell−ECM interactions are altered due to matrix degradation and reduced expression of matrix-binding integrins, preventing prosurvival integrin signaling and resulting in apoptosis [75]. This later phase of matrix degradation and apoptosis may be regulated, at least in part, by decreased production of WAP, a milk protein that can inhibit the activity of proteases that cleave laminin [69]. Decreased production of WAP by dying or dedifferentiated cells could relieve WAP-mediated protease inhibition, promoting protease activation and matrix degradation. This, in turn, would decrease the interaction of intact matrix molecules with integrins, decreasing prosurvival signaling. Furthermore, it is possible that integrins then interact with soluble matrix fragments, which is known to induce apoptosis in endothelial cells (see later) [53].

In endothelial cells, binding to intact matrix molecules in the basement membrane promotes survival and inhibits apoptosis, whereas binding to matrix fragments induces apoptosis [76]. For example, $\alpha_v\beta_3$ integrin interactions with intact matrix molecules play a crucial role in endothelial cell survival during embryonic angiogenesis. Indeed, disruption of these interactions with an antibody to $\alpha_v\beta_3$ inhibits the development of new blood vessels in the CAM by inducing endothelial cell apoptosis [77]. In contrast, $\alpha_v\beta_3$ binding to tumstatin, a proteolytic fragment of collagen IV, induces endothelial cell apoptosis, preventing angiogenesis and/or promoting vessel regression [78]. This interaction may promote apoptosis by interfering with normal integrin−ECM binding, thereby removing a critical survival signal. However, tumstatin may also promote apoptosis through a separate mechanism, such as via the recruitment and activation of caspase 8, as has been suggested previously for such soluble ligands [53]. Taken together, these findings suggest that disruption of cell−ECM interactions may lead to an increase in the expression or activation of proapoptotic molecules and may also lead to the removal of prosurvival signals, which then directly or indirectly cause apoptosis in endothelial cells.

Wound healing

Adhesion and migration

Early in the wound healing process, blood components and tissue factors are released into the wounded area in response to tissue damage, promoting both the coagulation cascade and platelet adhesion and activation, resulting in the formation of a clot consisting of platelets, cross-linked fibrin, fibronectin, and vitronectin, with lesser amounts of SPARC, tenascin, and thrombospondin [79]. Activated platelets, along with degranulating mast cells, release a number of cytokines and growth factors important in in regulating the wound healing process and initiating the next phase of wound healing, the inflammatory response. The fibrin-fibronectin clot has functions in addition to hemostasis, sequestering cytokines and growth factors while providing a temporary ECM that facilitates the adhesion and migration of multiple cell types, particularly leukocytes, into the wounded area [79].

Leukocyte adhesion, migration, and secretion of inflammatory mediators are regulated by their interactions with multiple molecules, some of which are ECM molecules [80]. After initial interactions between neutrophil and macrophage integrins with nonmatrix cell adhesion molecules ICAM and VCAM, these leukocytes interact with chemoattractant cytokines, or chemokines, associated with HSPG on the endothelial surface, in the endothelial basement membrane, and within the fibrin-based provisional matrix [80]. This chemokine binding to HSPG creates and maintains stable chemokine gradients critical for appropriate leukocyte recruitment, as shown by their defective recruitment by mutant chemokines lacking the ability to bind glycosaminoglycans, in mice deficient in heparan sulfate biosynthesis, and in mice overexpressing heparanase [80,81]. Leukocyte adhesion to and migration through the basement membrane and provisional matrix are also mediated by matrix interactions with matrix

receptors. Neutrophils bind several matrix molecules in the basement membrane, including fibronectin, vitronectin, laminin-511, laminin-411, and the proteoglycan lumican, and also bind fibrin and fibronectin in the provisional matrix [82]. Laminin-511 stabilizes the endothelium by binding endothelial integrin $\alpha_6\beta_4$ at hemidesmosomes and limits neutrophil movement through the endothelium to regions with low levels of laminin-511 [70,83]. In contrast, both laminin-411 and lumican are necessary for neutrophil extravasation, the former likely through integrin $\alpha_6\beta_1$ and the latter via β_2 integrin(s) [84,85].

After leukocytes leave the vasculature, they encounter the provisional matrix, which then regulates their adhesion, migration, and behavior. Integrin $\alpha_M\beta_2$ expressed by various inflammatory cells interacts with fibrin, urokinase plasminogen activator (uPA), and thrombospondin 4, inducing inflammatory cell adhesion and migration [86]. Binding of $\alpha_M\beta_2$ to uPA also promotes plasmin activation and thus fibrin degradation and removal of the provisional matrix [86]. In addition to the impact of integrin $\alpha_M\beta_2$ activation on matrix degradation, integrin $\alpha_M\beta_2$–ligand binding promotes proinflammatory cytokine production. In monocytes, fibrin binding induces expression of proinflammatory cytokines and chemokines, including IL-1β, IL-6, TNF-α, and several CC chemokines, and neutrophil binding to thrombospondin-4 induces secretion of the chemokine CXCL8 and the respiratory burst [86,87]. Many of these cytokines and chemokines can interact with matrix components, particularly HSPGs, to form a stable gradient that guides extravasated leukocytes to the injured area [80]. The presence of many matrix-degrading enzyme during this process ensures the generation of matrix fragments that also affect inflammation [5]. Hyaluronidases and reactive oxygen and nitrogen species can generate low molecular weight hyaluronan fragments, for example, which then activate TLRs like TLR4 in tissue macrophages and induce their release of proinflammatory cytokines, promoting adhesion and migration of additional inflammatory cells to the injured area [24]. As such, the types of ECM molecules and matrix fragments present in the injured area may greatly affect the inflammatory phase of wound healing.

This inflammatory phase overlaps with the next phase of wound healing, a proliferative phase that involves proliferation and migration of epithelial keratinocytes during reepithelialization as well as endothelial cells and fibroblasts during granulation tissue formation. In the reepithelialization phase of cutaneous wound healing, keratinocytes migrate beneath the fibrin-rich provisional matrix, likely due, at least in part, to the fact that these cells do not express the fibrin-interacting integrin $\alpha_V\beta_3$ [79,88]. The keratinocytes do express multiple receptors for fibronectin, collagen, tenascin, and vitronectin, including the integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$, and these receptor–matrix interactions, along with the activity of matrix-degrading MMPs and plasmin, promote keratinocyte migration and subsequent wound closure [70,79]. Keratinocyte migration requires the synthesis and deposition of laminin-332 and cellular fibronectin, as well as their interactions with multiple integrins [79]. Interactions between epithelial cells and ECM are also critical in the closure of other types of epithelial wounds. After wounding, retinal pigment epithelial cells exhibit a sequential pattern of ECM molecule deposition that is critical in the epithelial cell adhesion and migration associated with wound closure. Within 24 hours of wounding, these epithelial cells secrete fibronectin, followed shortly by laminin and collagen IV. If cell adhesion to these ECM molecules is blocked with either cyclic peptides or specific antibodies, the epithelial cells fail to migrate and close the wound, underscoring the importance of such interactions in wound closure [89,90].

Along with the deposition of new matrix molecules that facilitate cell adhesion and migration, proteases such as MMPs and plasmin are important in keratinocyte migration, facilitating migration by cleaving matrix molecules and cell–cell adhesion molecules that otherwise promote cell adhesion and prevent migration [9]. MMP-10, for example, may cleave laminin-332 and the α_6 subunit of integrin $\alpha_6\beta_4$ to allow deadhesion of keratinocytes from laminin-332, facilitating migration [91,92]. MMP-7 appears to cleave E-cadherin to allow cells to detach from each other and facilitate their migration, as keratinocytes in MMP-7-deficient mice exhibit both a lack of E-cadherin cleavage and migration defects [91]. In addition to direct cleavage of adhesion molecules or receptors by MMPs, proteases can also mediate the release of HB-EGF from HSPG, which then induces keratinocyte migration through the EGFR [9]. Therefore proteases can facilitate cell adhesion/deadhesion and migration using multiple mechanisms during reepithelialization.

During later stages of wound healing, macrophages and fibroblasts in the injured area deposit cellular fibronectin, tenascin-C, SPARC, proteoglycans, and collagens, which are important in the generation of the granulation tissue, a temporary connective tissue consisting of multiple types of ECM molecules and newly formed blood vessels [79]. These matrix molecules provide substrates for the migration of endothelial cells into the granulation tissue to form the wound vasculature, while also facilitating the recruitment of fibroblasts, myofibroblasts, and lymphocytes whose migration is stimulated by a variety of chemokines produced by tissue fibroblasts and macrophages [93]. Many chemokines have been characterized in multiple species, including humans, other mammals, and even birds, and have been grouped into a large superfamily which is further subdivided based upon the

position of the N-terminal cysteine residues. These chemokines, along with cell−ECM interactions, are critical in the adhesion and chemotaxis/migration of the cells that ultimately enter the wounded area and generate the granulation tissue [93]. One prototypical chemokine, CXCL8 (IL-8), has several functions that are important in wound healing. Many of these functions have been elucidated in studies performed in the chick model system using the chicken version of CXCL8, chCXCLi2 (cCAF/cIL-8) [94]. After wounding, fibroblasts in the injured area produce large quantities of chCXCLi2, most likely resulting from their stimulation by thrombin, a coagulation enzyme activated upon wounding that is known to induce fibroblasts to express and secrete chCXCLi2. The initial rapid increase in chCXCLi2 generates a gradient that chemoattracts neutrophils, a finding that has since been replicated with human neutrophils and CXCL8 [80,95]. CXCL8 derived from tissue fibroblasts and macrophages, along with the additional CXCL8 secreted from the endothelial cells of the wound vasculature and bound to various matrix components of the granulation tissue, participates in granulation tissue formation by stimulating angiogenesis and matrix deposition [96,97]. Therefore CXCL8 not only functions in the inflammatory phase of wound healing by serving as a leukocyte chemoattractant but also plays an important role in granulation tissue formation.

Angiogenesis is a critical part of granulation tissue formation and relies heavily upon cell−ECM interactions. The localization of the matrix molecules and the proteases that degrade them have critical roles in this process. In some cases, interaction of an angiogenic factor or its receptor with a matrix molecule is important in ligand−receptor interactions and/or downstream signaling, whereas in others, matrix fragments signal differently from the parent matrix molecule [5,14]. As mentioned earlier, many extracellular signaling molecules, including CXCL8, VEGF, and FGF, bind HSPG, which can alter their diffusion and interaction with their receptors [8,50]. In addition to HSPG, however, FGF and VEGF signaling is heavily influenced by the presence of specific matrix molecules and integrins. Integrin $\alpha_v\beta_3$ is important in VEGFR2 and FGFR activation and angiogenesis induced by VEGF and FGF, respectively, and integrin $\alpha_v\beta_5$ is also involved in VEGF-induced angiogenesis [14]. In the case of VEGF/VEGFR2 signaling, an integrin $\alpha_v\beta_3$ ligand, vitronectin, promotes the interaction of this integrin with VEGFR2, enhances VEGF-induced VEGFR2 signaling, and promotes endothelial cell migration [98,99]. Similarly, fibronectin simultaneously binds VEGF and integrin $\alpha_5\beta_1$, promoting VEGF-induced endothelial cell migration, whereas collagen I binding to β_1 integrins instead inhibits signaling downstream of VEGFR2 [98,100]. VEGF can also bind integrin $\alpha_9\beta_1$ directly, promoting VEGFR2-mediated signaling and ultimately endothelial cell adhesion and migration [100,101]. Similarly, FGF2 can bind directly to integrin $\alpha_v\beta_3$, which is necessary for endothelial cell migration and angiogenesis induced by FGF2. FGF2 mutants defective in integrin binding were unable to promote endothelial cell migration, tube formation on Matrigel, and angiogenesis in vivo [102]. Taken together, these results underscore the importance of the microenvironment in regulation of angiogenesis induced by VEGF and FGF2.

Along with the matrix proteins in the microenvironment, protease activity is needed for the endothelial cells to degrade and then migrate through the basement membrane and surrounding connective tissue during angiogenesis [5,103]. These proteases can regulate endothelial cell adhesion and migration during angiogenesis by releasing matrix-bound factors, as mentioned earlier, and also by generating functional matrix fragments and exposing previously concealed matricryptic sites [5]. Some of these "matricryptins" promote cell migration and angiogenesis. For example, a matricryptic site in collagen IV exposed by MMP-9-mediated proteolysis changes integrin binding from $\alpha_1\beta_1$ to $\alpha_v\beta_3$, and blocking this site with an antibody or inhibiting integrin $\alpha_v\beta_3$ prevents endothelial cell adhesion and migration in vitro and angiogenesis in vivo [100]. Similarly, a fragment of collagen 1 that is released by MMP-mediated cleavage promotes angiogenesis [5]. However, the contribution of matricryptins in the regulation of angiogenesis is complicated by the fact that although some matricryptins promote angiogenesis, many others inhibit angiogenesis, including fragments derived from collagen XVIII (endostatin) and collagen IV (canstatin, tumstatin, and arresten). Endostatin, canstatin, and arresten inhibit endothelial cell migration and tube formation [78]. These matricryptins may exert their inhibitory effects by binding VEGFR2 (endostatin) and/or integrin $\alpha_v\beta_3$ (endostatin, canstatin, and tumstatin), both of which are essential for VEGF-induced cell migration and angiogenesis, thereby blocking signaling downstream of the individual receptor(s) and the substantial cross talk that occurs between them [5,78].

Proliferation

After cutaneous wounding, keratinocytes alter their proliferation and migration in order to close the wound, a process known as reepithelialization. As this process occurs, the cells at the edge of the wound migrate, whereas the cells away from the wound proliferate to provide the cells needed to cover the wounded area. The proliferative state of the keratinocytes may be sustained by interactions with the ECM of the remaining or nascent basement membrane. ECM derived from the basal lamina, when present in a dermal wound model, can maintain keratinocytes in a

proliferative state for several days. As mentioned before, the proliferation of the basal layer of keratinocytes needed to replace the upper keratinocyte layers in uninjured skin requires components of the epithelial basal lamina, including fibronectin, with potential contributions of collagen IV and laminin-511, which promote proliferation in vitro, and the laminin receptor $\alpha_6\beta_4$ is necessary for keratinocyte proliferation [72,73]. In addition, integrins appear to regulate keratinocyte proliferation. For example, fibronectin-binding integrins $\alpha_5\beta_1$ and $\alpha_v\beta_6$ are upregulated in keratinocytes after wounding, suggesting a potential mechanism whereby fibronectin may prevent terminal differentiation and/or promote proliferation of keratinocytes during reepithelialization [93]. Furthermore, expression of integrin α_9 is increased upon wounding, and keratinocytes in mice lacking epithelial integrin α_9 or β_1 exhibit reduced proliferation [14,104]. These results suggest a role for integrin $\alpha_9\beta_1$, a receptor for several matrix molecules, including laminin, tenascin-C, and emilin, in keratinocyte proliferation [104]. Complicating our understanding of integrin $\alpha_9\beta_1$-mediated adhesion in this process, emilin-1-deficient keratinocytes exhibit excessive proliferation during reepithelialization in vivo and in cultured cells in vitro, the latter of which is dependent upon α_9 and α_4 integrins [105]. As such, the specific integrins and their specific ligands present at different stages of the reepithelialization process may either promote keratinocyte proliferation or limit such proliferation.

While reepithelialization progresses, the granulation tissue begins to form, a process involving many ECM molecules, including cellular fibronectin, collagen III, collagen I, and hyaluronic acid, and multiple proliferating cell types, notably the endothelial cells of the wound vasculature and fibroblasts. Fibronectin, fibronectin fragments, laminins, collagen VI, SPARC, tenascin-C, and hyaluronan provide a context that facilitates fibroblast and endothelial cell proliferation [106]. Fibroblasts lacking the collagen receptor DDR2 exhibit reduced proliferation in vitro, and the granulation tissue of $DDR2^{-/-}$ wounds contain fewer fibroblasts, likely due to decreases in their proliferation and/or migration, suggesting that some matrix molecules provide cues necessary for cell proliferation [33]. Matrix molecules can also promote proliferation directly, as shown by the ability of the EGF-like domains of tenascin-C to promote fibroblast proliferation in an EGFR-dependent manner [54]. Growth factors themselves can require specific ECM molecules to induce proliferation, as is seen in the fibronectin requirement for TGFβ1-mediated fibroblast proliferation [107]. In addition to fibroblast proliferation in the granulation tissue, endothelial cells proliferate during angiogenesis of this tissue, a process that is dependent upon growth factors, such as FGFs and VEGFs, and their interactions with matrix molecules. Signaling induced by both FGFs and VEGFs is enhanced by HSPG, as discussed earlier, while VEGF binding to fibronectin or vitronectin enhances its effect on endothelial cell proliferation [50,99]. In contrast, certain ECM molecules and/or proteolytic fragments can inhibit proliferation. The matricellular proteins SPARC inhibits VEGF- and FGF2-induced proliferation, indicating that interactions between growth factors and ECM can also be inhibitory [8]. Furthermore, SPARC and decorin, as well as peptides derived from SPARC, decorin, collagen IV (tumstatin), and collagen XVIII (endostatin) are antiangiogenic due to their inhibitory effects on endothelial cell proliferation [29,103].

Differentiation

As the granulation tissue forms, some of the fibroblasts within the wounded area differentiate into myofibroblasts, contractile cells that express the protein α-smooth muscle actin and secrete a number of matrix molecules, including fibronectin and collagens I and III [93]. Myofibroblast differentiation is regulated by cytokines and growth factors, mechanical tension, and interactions with multiple matrix molecules, including proteoglycans, cellular fibronectin, interstitial collagen, and hyaluronan. One of the best characterized growth factors that induces myofibroblast differentiation is TGFβ1, which is initially secreted in an inactive, latent state and must be activated either through a change in conformation or proteolytic cleavage to an active form able to bind its receptor [14]. Release of active TGFβ1 is insufficient for receptor activation, however, as several proteoglycans, including decorin, can bind active TGFβ1 and prevent it from binding its receptor [8]. For TGFβ1 to bind and activate its receptor thus also requires proteolytic degradation of these sequestering proteoglycans. In contrast, binding of the proteoglycan betaglycan to TGFβ1 has different effects on downstream signaling depending upon whether betaglycan is membrane-associated or soluble. Membrane-bound betaglycan presents TGFβ1 to its receptor and facilitates downstream signaling, whereas soluble betaglycan competes with membrane betaglycan for TGFβ1 binding and therefore prevents downstream signaling [62]. Changes in the presence or abundance of proteoglycans and enzymes that degrade them can thus influence the availability of active TGFβ1 in the healing wound, impacting its ability to induce myofibroblast differentiation and contraction of the granulation tissue.

Matrix molecules other than proteoglycans are also important in myofibroblast differentiation. TGFβ1-induced differentiation requires the Fibronectin Extra Domain A (EDA)-containing form of cellular fibronectin and the binding of this EDA domain to integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ [93]. Interstitial collagens, in conjunction with mechanical tension exerted by integrin-mediated adhesion to

collagens, also participate in the differentiation process. Fibroblasts cultured on relaxed collagen gels fail to differentiate, whereas fibroblasts grown on stiffened collagen matrices exhibit myofibroblast characteristics [108]. This finding, coupled with data demonstrating increased myofibroblast differentiation when splints are used to increase mechanical tension in wounds, suggest a role for mechanical tension in myofibroblast differentiation in vivo [109]. Tensile stress may regulate cell signaling and cell−matrix interactions by revealing cryptic sites in intact matrix molecules, by changing the conformation of latent TGFβ1 to facilitate receptor binding, and by altering signaling downstream of matrix receptors [93,108]. For example, mechanical stress could expose the EDA domain of fibronectin needed for TGFβ-induced myofibroblast differentiation, and several RGD-binding integrins can interact with the RGD motif of the latency-associated peptide of TGFβ1 and either induce the appropriate change in conformation needed to bind the receptor or present the latent complex to MMPs to facilitate proteolytic cleavage and release of active TGFβ1 from the latent complex [108]. Tensile stress exerted on the cell by the matrix may also induce the formation of stress fibers that exert intracellular tension on integrins interacting with these matrix molecules, promoting the formation of strong adhesions needed for wound contraction and closure [108]. Mechanical strain may further alter myofibroblast differentiation and function by altering signaling downstream of integrins subjected to increased mechanical tension while adhering to their matrix ligands [108].

Apoptosis

Late in the wound healing process, the granulation tissue undergoes remodeling to form scar tissue. This remodeling phase is characterized by decreased tissue cellularity due to the disappearance of multiple cell types, including fibroblasts, myofibroblasts, endothelial cells, and pericytes, and by the accumulation of ECM molecules, particularly interstitial collagens. The observed reduction in cell numbers during the remodeling phase occurs largely via apoptosis, and many of these apoptotic cells are endothelial cells and myofibroblasts, as shown by studies using in situ DNA fragment end-labeling in conjunction with transmission electron microscopy [110]. Apoptosis of fibroblasts and myofibroblasts may be important in preventing excessive scarring and facilitating the resolution of wound healing. Indeed, reduced fibroblast and myofibroblast apoptosis in keloids and hypertrophic scars is associated with increased matrix deposition and scarring, whereas inducing myofibroblast apoptosis in a mouse scleroderma model reduced fibrosis [109,111]. Myofibroblast apoptosis is regulated by mechanical tension and occurs after the myofibroblasts contract to close the wound, as the tension of the tissue decreases. Using a splint to maintain tension in a healing wound inhibits myofibroblast apoptosis, whereas the release of tension in this model promotes apoptosis, implicating mechanotransduction in apoptosis regulation [109]. This may be mediated by changes in signaling through the collagen receptor DDR2 after release of tension. Though more work must be done to understand the relationship between DDR2, collagens, and mechanotransduction, a recent study found that $DDR2^{-/-}$ myofibroblasts undergo more apoptosis in a lung fibrosis model than wild-type myofibroblasts, coinciding with reduced fibrosis [112]. Taken together, cell/matrix interactions and associated mechanotransduction regulate myofibroblast apoptosis after wound contraction.

Signal transduction events during cell−extracellular matrix interactions

As discussed before, ECM molecules are capable of interacting with a variety of receptors. Such interactions activate signal transduction pathways within the cell, altering levels of both gene expression and protein activation, ultimately changing cellular outcomes in cell adhesion, migration, proliferation, differentiation, and death. The signaling pathways linked to these specific outcomes have been studied for many of the ligand−receptor interactions, particularly those involving integrins [14]. In the case of integrins, it is important to remember that these receptors can participate in both “outside-in” and “inside-out” signaling [113,114]. Outside-in signaling occurs when an extracellular ligand binds the receptor and initiates intracellular signaling, and in inside-out signaling, intracellular signaling increases the affinity of the receptor for its ligand. Binding of the receptor to the ligand then initiates outside-in signaling. Unless otherwise indicated, the signaling events discussed next refer to outside-in signaling.

Based upon the many studies that investigate matrix-induced signaling, we postulate the existence of three categories of cell−ECM interactions, namely, type I interactions that are involved in adhesion and migration, type II interactions involved in proliferation, differentiation, and survival, and type III interactions involved in apoptosis (Fig. 6.2). Although these three categories of cell signaling in cell−ECM interactions may not be exhaustive of the general types of adhesions that occur during development and wound healing, they encapsulate some of the best understood interactions and their associated signaling events. Each category has its place in many developmental and repair events, and they may operate in sequence to promote the cell survival, adhesion, migration, differentiation, and apoptosis needed to generate tissues and organs, and to promote their repair and regeneration following injury.

Type I interactions are generally mediated by integrin and proteoglycan receptors and are important in the

adhesion/deadhesion processes that accompany cell migration (Fig. 6.2A). Integrins interact with ligands outside the cell, and this triggers intracellular signaling through proteins associated with integrins in integrin adhesion complexes [115]. These integrin adhesion complexes include proteins that connect the integrins indirectly with the actin microfilaments, those involved in signal transduction, and others involved in mechanotransduction. For example, the microfilaments interact with the cytoplasmic domain of integrin β subunits through the structural proteins talin and α-actinin, transduce mechanical signals through vinculin and talin, facilitate signaling through focal adhesion kinase (FAK) and paxillin, and regulate the actin cytoskeleton via vasodilator-stimulated phosphoprotein and zyxin [115,116]. However, many of these proteins have overlapping roles and ultimately work together to control cell adhesion and migration. Binding of an integrin to its ligand triggers autophosphorylation of FAK on tyrosine 397 (PY397), which then serves as the binding site for the SH2 domain of the c-Src tyrosine kinase [117]. This kinase subsequently phosphorylates FAK itself at position 925, increasing its activity while facilitating FAK binding to downstream signaling molecules, including Grb2/Sos, PI3K, and $p130^{Cas}$ [104]. This autophosphorylation and activation of FAK is enhanced when cells adhere to stiff matrices when compared with adhesion to softer matrices, and the phosphorylation of $p130^{Cas}$ downstream of FAK/Src signaling only occurs on stiff matrices, suggesting the importance of mechanosensation in FAK/Src signal transduction [118,119].

After FAK phosphorylation at Y925, it interacts with the Grb2/Sos complex, promoting the downstream activation of Ras and the MAP kinase cascade, which may be involved in cell adhesion/deadhesion and migration events [117,120]. Beyond Ras and the MAPK cascade, the FAK/Src complex activates a number of downstream signaling molecules necessary for adhesion and migration, including paxillin, $p130^{Cas}$, tensin, and vinculin. Paxillin regulation

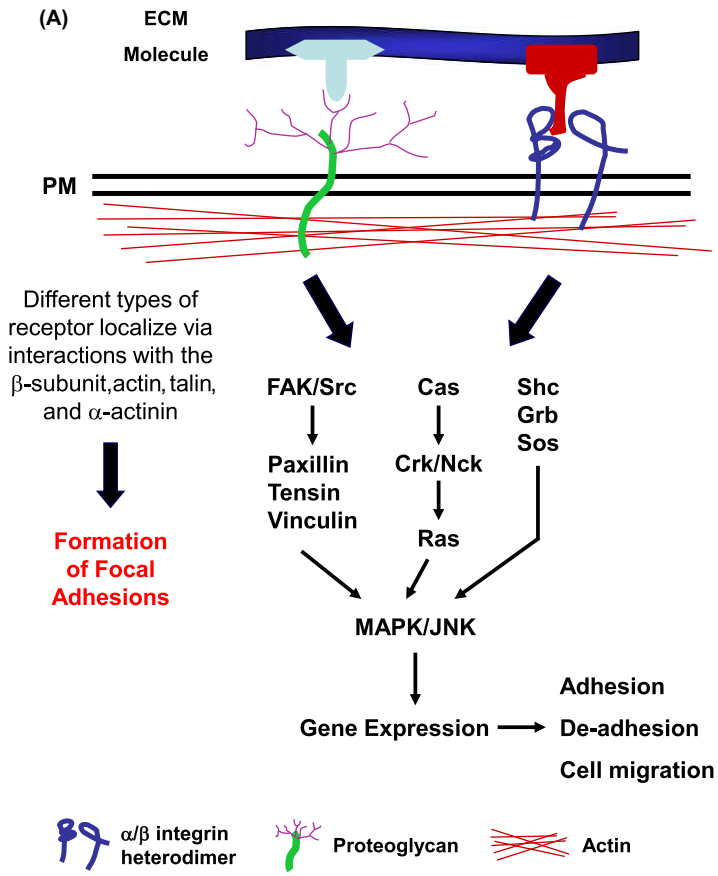

FIGURE 6.2 Schematic diagram of cell–ECM interactions present during healing and regenerative responses. Type I (A), Type II (B) and Type III (C). Such interactions between the ECM receptors and their respective ligands initiate signal transduction cascades culminating in a variety of cellular events important in repair and regeneration, including changes in cellular adhesion and migration and altered rates of proliferation and apoptosis. The presence and/or extent of such changes may influence the balance of repair and regenerative responses to favor one outcome over another; thus interventions that alter ECM signaling events may shift this balance to favor tissue regeneration and thus decrease scarring. *ECM*, Extracellular matrix.

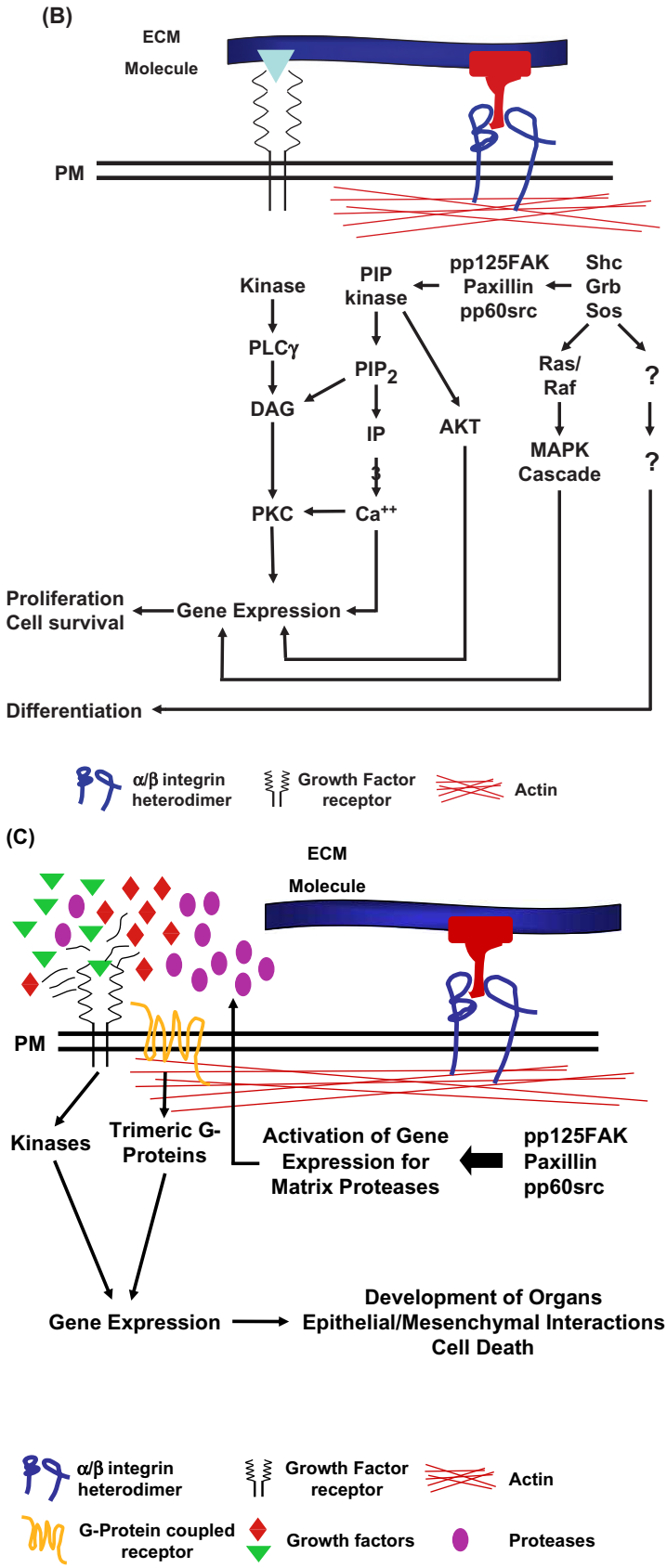

FIGURE 6.2 (Continued)

by FAK/Src is necessary for integrin-mediated signaling and motility, as shown by the reduced migration and decreased phosphorylation/activation of multiple signaling molecules in paxillin-deficient fibroblasts, coupled with the impaired migration seen in cells overexpressing a paxillin mutant that could not be phosphorylated by FAK/Src [121]. Both phosphorylated paxillin and $p130^{Cas}$ bind the Crk/DOCK180/ELMO complex, activating DOCK180, a guanine nucleotide exchange factor (GEF) that activates Rac1, while paxillin recruits another Rac1/Cdc42 GEF, β-PIX, thereby inducing lamellipodia formation and cell migration [117,122,123]. Src-mediated signaling also activates another Rac GEF, Vav2, along with a Rho GTPase-activating protein (GAP) that inhibits RhoA [123]. Similarly, members of the tensin family, which bind both actin microfilaments and integrins and are phosphorylated downstream of FAK/Src signaling, interact with and activate DLC1, a GAP inhibiting RhoA, and Dock5, a GEF-activating Rac1 [124]. Collectively, this signaling increases the activity of Rac1, promoting the formation of lamellipodia at the leading edge of migrating cells, while decreasing activity of RhoA and thereby causing deadhesion, ultimately facilitating directional migration.

To this point, we have discussed the roles of FAK and Src in outside-in, traditional receptor-mediated signal transduction. However, these kinases can participate in inside-out signaling as well. FAK/Src signaling stimulates integrin activation, cell adhesion to matrix molecules, and focal adhesion strengthening [125]. This integrin activation is mediated by members of the talin and kindlin families, which are recruited to the integrins at nascent adhesions in a FAK-dependent manner [114]. Indeed, integrin activation and integrin-mediated adhesions are inhibited in cells lacking talin or kindlin and in cells whose talin cannot bind integrins [126,127]. Integrin activation and matrix binding then initiates outside-in signaling, promoting FAK/Src activity and leading to Rac1 activation and RhoA inhibition. Like tensin, kindlin may participate in this outside-in signaling after integrin activation by interacting with and activating DLC1, leading to deadhesion downstream of RhoA inactivation [114]. Taken together, these findings suggest a continuous cycle of outside-in and inside-out signaling, with outside-in signaling triggering protrusion formation and deadhesion and inside-out signaling increasing adhesion formation, which may function in sequence to promote cell migration.

Integrins can also cooperate with growth factor receptors to promote downstream signaling, as described earlier. c-Src activated downstream of VEGFR2 phosphorylates β_3 integrin and increases adhesion of integrin $\alpha_v\beta_3$ to vitronectin. Vitronectin-induced integrin activation promotes its interaction with VEGFR2 in a Src-manner, ultimately increasing endothelial cell migration downstream of VEGFR2 signaling [98,99,128]. This VEGF-induced endothelial cell migration is also dependent upon FAK, suggesting that signaling downstream of VEGFR2 and integrin $\alpha_v\beta_3$ work together to regulate and coordinate cell migration during angiogenesis [129].

Matrix receptors other than integrins, such as proteoglycans, CD44, the ERC, RHAMM, and DDR1/2, are also important in cell adhesion and migration. Syndecans are proteoglycans that can function as coreceptors, associating with matrix molecules and integrins to promote cell adhesion and migration [20]. Syndecan-4, for example, can bind the heparin-binding domain of fibronectin and the fibronectin receptor integrin $\alpha_5\beta_1$, activating PKC and Rac activation and RhoA inactivation downstream of PKC [130]. This promigratory regulation of Rho GTPases is coupled with a loss of integrin-mediated adhesions, as syndecan-4-induced PKC and RhoG promote integrin $\alpha_5\beta_1$ internalization and degradation after fibronectin binding, thus promoting the deadhesion needed for cells to migrate [113]. In addition to interacting with matrix molecules and integrins to facilitate or alter downstream signaling, syndecans are also present in complexes including integrins, matrix molecules, growth factors, and growth factor receptors. In these complexes, syndecans can promote growth factor binding and activation of its receptor, as is the case for FGF2 and VEGF, prevent receptor binding until proteolytic release of the ligand, as in the case for HB-EGF, and may contribute to cell migration downstream of these growth factors [20].

Receptors other than syndecans and integrins can also promote cell migration, including the hyaluronan-binding receptors CD44, RHAMM, and TLRs. As described in more detail before, native hyaluronan has a high molecular weight that interacts with CD44 and RHAMM but not TLRs, whereas smaller hyaluronan fragments generated by hyaluronidases or reactive oxygen or nitrogen species interact with TLRs [21,23,24]. High molecular weight hyaluronan binding to CD44 induces phosphorylation of its intracellular domain and activation of c-Src, resulting in fibroblast migration [131]. RHAMM, another hyaluronan receptor, cooperates with CD44 to facilitate fibroblast migration by regulating ERK1/2 activation downstream of CD44, and in smooth muscle cells, hyaluronan induces cell migration downstream of RHAMM via PI3K and Rac [132]. In contrast to high molecular weight hyaluronan, low molecular weight hyaluronan induces inflammation, largely through its activation of TLRs and downstream activation of NFκB and induction of cytokine expression [23,131]. As such, low molecular weight hyaluronan induces inflammatory cell migration in an indirect manner via increasing levels of proinflammatory cytokines.

In addition to hyaluronan fragments, fragments of other matrix molecules can induce cell adhesion and migration through their interactions with the ERC and growth factor receptors. Fragments of elastin, laminin,

and fibrillin bind the ERC, which promotes matrix deposition and thereby increases cell adhesion, while also inducing cell migration, likely through activation of MAP kinase cascades [25]. Similarly, several matrix molecules with EGF-like domains bind and activate the EGFR, either as part of the intact matrix molecule, or, more likely, following release of the EGF-like domains after proteolytic degradation, and promote cell migration downstream of the EGFR [133].

Signaling downstream of the collagen receptors DDR1 and DDR2 can induce migration in some cell types, while decreasing cell migration in others. Therefore the signaling and cellular outcome is dependent upon the cellular context. DDR1 activation by collagen inhibits epithelial cell adhesion and migration induced by integrin $\alpha_2\beta_1$ ligation, likely through recruitment of the phosphatase SHP-2 but promotes migration of smooth muscle cells and T cells in response to collagen [134]. Inhibition or knockdown of DDR1 inhibited migration of Th17 cells through a collagen matrix, and inhibition of DDR1 inhibited Th17 cell recruitment to the chemokine CCL20 in vivo, suggesting the importance of this collagen receptor in T-cell migration [134]. DDR1-mediated Th17 migration in vitro requires activation of RhoA and its effector ROCK, as well as the activation of ERK downstream of Rho/ROCK signaling [135]. Collagen-induced fibroblast migration is instead mediated by DDR2, as shown by the reduced migration of DDR2$^{-/-}$ fibroblasts through Matrigel, which was rescued by reexpression of DDR2 in these cells [33,134].

DDR2-mediated migration in this context requires MMP2, presumably to promote invasion through the Matrigel [33]. In contrast, DDR2-mediated fibroblast migration was dependent upon JAK2 and ERK [33]. Complicating our understanding of DDR1 and DDR2 signaling is the cross talk linking these receptors to collagen-binding integrins, which are expressed in the same cells and bind the same ligands, and DDR1/2 signaling promotes integrin binding to collagens [134]. As such, these receptors likely work together to mediate cell adhesion and migration.

Type II interactions involve processes in which the matrix−receptor interactions, in conjunction with growth factor or cytokine receptors, affect proliferation, survival, differentiation, and/or maintenance of the differentiated phenotype (Fig. 6.2B). These cooperative effects may occur in a direct manner, for example, by the direct interaction of EGF-like repeats present in certain ECM molecules with the EGF receptor, thereby promoting cell proliferation [12]. However, more current evidence supports the importance of indirect cooperative effects, particularly with regards to the anchorage dependence of cell growth. S-phase entry, even when growth factors are present, requires the interaction of cells with a substrate, while detachment of cells from matrix promotes a type of apoptosis called anoikis, underscoring the critical role of cell−ECM adhesion in cell survival and proliferation [136].

The prosurvival function of matrix interactions is largely mediated by integrins and their downstream activation of FAK. In epithelial cells, FAK/Src complexes promote cell survival through PI3K-induced Akt activation [137,138]. Signaling downstream of Akt then increases levels of antiapoptotic Bcl family members while decreasing levels of proapoptotic Bcl family members, inhibiting apoptosis and resulting in cell survival [138]. A similar mechanism may promote cell survival following CD44 binding to hyaluronan, which also activates PI3K and Akt [139].

While cell survival signaling induced by matrix binding to receptors like integrins and CD44 is necessary but not sufficient to induce proliferation, integrin signaling can promote cell proliferation either alone or in conjunction with growth factor signaling. Integrin-induced activation of the Rac/JNK pathway can induce cell proliferation by stimulating expression of cyclin D and by promoting the degradation of p21, a cell cycle inhibitor [140]. In fibroblasts, FAK/Src signaling induces Rac activation downstream of p130CAS, which then promotes cell survival and proliferation via the JNK pathway [117]. ERK1/2, which are, like JNK, MAP kinases, can be activated by integrin ligation. However, when activating ERK1/2, integrin ligation activates the Src kinase Fyn, which binds the Shc adaptor protein, leading to the recruitment of Grb2/Sos and downstream activation of the Ras/ERK pathway [136]. This ultimately induces phosphorylation of the transcription factor Elk-1, which then activates genes important in cell cycle progression [136,141].

Beyond the direct impact of integrin signaling on cell proliferation, integrins can facilitate cell survival and proliferation in conjunction with growth factors, their receptors, and their associated signaling [14]. Matrix molecules and matrix receptors can directly interact with growth factors to affect downstream signaling. Fibronectin and collagen both interact with VEGF. Binding of VEGF to fibronectin increases VEGFR activation and endothelial cell proliferation, whereas binding to collagen activates a phosphatase and thus decreases VEGFR activity [98]. HSPG can interact with many growth factors, including FGF and VEGF, limiting their diffusion, sequestering them until enzyme-mediated release, or promoting their receptor binding [50]. FGF and VEGF binding to HSPG promote their interactions with their respective receptors and activate mitogenic signaling [8]. Matrix molecules can also bind growth factor receptors and activate them directly, as in the binding and activation of the EGFR by the EGF-like domains of laminin and tenascin-C, which may promote cell proliferation [133]. In contrast, decorin binding to the EGFR promotes EGFR internalization and thus inhibits mitogenic signaling downstream of this receptor [11].

Integrins may interact directly with growth factors and/or their receptors to regulate downstream signaling. Multiple VEGF isoforms bind and activate integrin $\alpha_9\beta_1$, which, along with VEGFR2, is necessary for VEGF-induced paxillin and Erk phosphorylation and angiogenesis [18]. Similarly, integrin $\alpha_V\beta_3$ interacts with both IGF-1 and FGF2 and is required for proliferation induced by both growth factors [136]. Integrins can also interact with growth factor receptors to regulate downstream signaling, as suggested by data showing that cell–ECM interactions are critical for the efficient and prolonged activation of MAP kinases by growth factors, likely participating in MAPK-induced proliferation [142]. Indeed, several growth factor receptors interact with integrins, including VEGFR2 and EGFR, and integrin-mediated adhesion can regulate growth factor-induced proliferation [136]. Angiogenesis induced by the growth factors FGF2 and VEGF requires the presence and activation of integrin $\alpha_V\beta_3$ and, in the case of VEGF, integrin $\alpha_V\beta_5$ [14]. Integrin signaling and FAK are necessary for activation of Erk1/2 by both FGF2 and VEGF, though Erk1/2 activation by FGF2/$\alpha_v\beta_3$ is dependent upon Pak-1-induced Raf phosphorylation but not Ras, while Erk1/2 activation by VEGF/$\alpha_v\beta_5$ requires Ras [143]. As it induces cell proliferation, FGF2-induced Pak-1 also inhibits apoptosis and thereby increases cell survival through its downstream regulation of apoptosis signal-regulating kinase 1 [14]. Once growth factor signaling is initiated, this signaling can, in turn, activate associated integrins and promote matrix adhesion. For example, VEGFR2 signaling induces phosphorylation of integrin β_3, leading to activation of FAK and p38 as well as VEGFR2-integrin binding and enhanced VEGFR2 signaling [98].

Interactions among growth factors, matrix molecules, and their receptors are not limited to integrins, however. Activation of several nonintegrin matrix receptors, including the ERC, CD44, TLRs, and discoidin domain receptors, are associated with cell survival and proliferation. Activation of the ERC by elastin fragments induces smooth muscle cell proliferation through activation of the MAPK cascade [144]. However, signaling through the ERC can inhibit proliferative signaling if the ERC neuraminidase subunit desialylates growth factor receptors, which interferes with ligand binding and downstream signaling [145]. These different effects of the ERC on cell proliferation, coupled with the ability of elastin-derived peptides to bind integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ as well as the ERC, complicates our understanding of elastin-derived peptides on cell proliferation [25]. Hyaluronan, which, like the ERC, binds multiple receptors, leads to Erk activation and endothelial cell proliferation through CD44, though high molecular weight hyaluronan more commonly inhibits cell division [23,132]. Low molecular weight hyaluronan fragments can activate CD44, inducing Erk activation, cyclin expression, and smooth muscle cell proliferation, although these hyaluronan fragments can also induce epithelial cell survival downstream of TLRs-induced NFκB [23,146]. Another nonintegrin receptor, DDR2, is important in cell proliferation, as $DDR2^{-/-}$ fibroblasts and chondrocytes proliferate less than wild-type fibroblasts, which could be explained, at least in part, by the ability of DDR2 to induce fibroblast survival through activation of the PDK1/Akt pathway [33,112]. Taken together, these examples suggest roles for nonintegrin matrix receptors in cell survival and proliferation, though the associated signaling pathways are not completely defined.

Like cell survival and proliferation, differentiation of many cell types, including keratinocytes, endothelial cells, and fibroblasts, is regulated by cell interactions with ECM molecules, hormones, and growth factors. For example, hyaluronan induces keratinocyte differentiation in vitro in a CD44-dependent manner, and CD44 deficiency inhibits keratinocyte differentiation in vivo, suggesting a role for epidermal hyaluronan in this process [147]. Complicating the picture of hyaluronan function in this differentiation process, hyaluronidase treatment increases the rate of keratinocyte differentiation, potentially due to the low molecular weight hyaluronan generated by hyaluronidase treatment, which has different receptor-binding properties than intact, high molecular weight hyaluronan [148]. Binding to components of the basement membrane negatively regulates keratinocyte differentiation in the basal layer of the epidermis, likely due, at least in part, to fibronectin interactions with the β_1 integrins also needed to repress differentiation [70,71].

During angiogenesis, endothelial cells undergo a differentiation process to generate the mature vasculature. Although in vitro models of this complex process are incomplete, they provide clues regarding the ability of the matrix to regulate this process. One early model involved the growth of endothelial cells on a basement membrane-like matrix mixture, Matrigel, which then formed capillary-like tubes, suggesting that one or more of the matrix molecules in this mixture could induce differentiation [47]. As mentioned before, laminins are present in large amount both in Matrigel and in basement membranes of blood vessels in vivo, though laminin-111 predominates in Matrigel and laminin-411 in basement membranes [149]. Whereas the binding of fibronectin to integrin $\alpha_5\beta_1$ in endothelial cells leads to cell proliferation, laminin binding to integrin $\alpha_2\beta_1$ in these cells promotes the formation of capillary-like structures, underscoring the importance of laminin-mediated adhesions in the formation of these structures in vitro [48,136]. Downstream molecules needed to generate the capillary-like tubes include integrin-linked kinase (ILK), which, when overexpressed, rescues capillary-like tube formation in the absence of ECM molecules, while expression of a dominant negative version of ILK blocks tube formation even when ECM and VEGF are present

[150,151]. More recent experiments found that ILK is necessary for the development of capillary-like structures in embryoid bodies derived from $ILK^{-/-}$ embryonic stem cells, further implicating ILK in this process [152]. Once nascent vessels are formed, contractile smooth muscle cells called pericytes are recruited to stabilize the endothelium and promote synthesis of the basement membrane [93]. Pericyte differentiation relies upon growth factors, including TGFβ and PDGF-BB, in conjunction with cell−matrix interactions. Some of these required matrix interactions are needed to activate TGFβ from its latent form, as described before [63]. However, pericytes in a mural cell-specific integrin β_1 knockout exhibited impaired differentiation, resulting in defective vessel stabilization and maturation [153]. Because various integrins are important for TGFβ activation, the role of integrin-mediated matrix adhesion in pericyte differentiation remains unclear.

Like pericytes, myofibroblasts are contractile cells differentiating from precursor cells, in this case, fibroblasts, in response to TGFβ signaling. TGFβ-induced myofibroblast differentiation requires activation of TGFβ from its latent form, adhesion of cells to the EDA domain of fibronectin, and the presence of mechanical tension, which may facilitate activation of TGFβ and/or reveal cryptic sites in matrix molecules to promote receptor binding [93,108]. The specific integrins involved in TGFβ activation include integrins $\alpha_v\beta_5$, $\alpha_v\beta_6$, and β_1 subunit-containing integrins [154,155]. Integrin $\alpha_4\beta_7$ may also participate in the differentiation process by binding to the EDA domain of fibronectin, leading to downstream activation of FAK, PI3K, and associated signaling pathways [6]. FAK may also be activated by mechanotransduction from matrices under tension, which is also important in myofibroblast differentiation [6,106]. Evidence demonstrating the need for ILK and Akt in TGFβ-induced myofibroblast differentiation further suggests a role for signaling downstream of integrin activation in this process [156]. Interestingly, a nonintegrin, EDA fibronectin-binding receptor may also facilitate myofibroblast differentiation, as TGFβ-induced myofibroblast differentiation in vitro requires the TLR TLR4 [157].

Type III interactions primarily involve processes leading to apoptosis (Fig. 6.2C). Typically, cell−matrix adhesions promote cell survival, whereas normally adherent cells that fail to interact with matrix molecules undergo anoikis, a form of cell death. Anoikis results from a lack of prosurvival FAK/Src-induced PI3K/Akt or Erk signaling, coupled with proapoptotic signaling that inhibits antiapoptotic Bcl family members, increases expression/activity of proapoptotic Bcl family members, and recruits and activates caspases [158]. Therefore inhibition of these prosurvival, antiapoptotic signaling pathways downstream of cell deadhesion leads to apoptosis. This does not, however, explain why the presence of unligated integrins can promote apoptosis even when other integrins adhere to their matrix ligands. Interestingly, these unligated integrins recruit procaspase-8, which subsequently becomes activated to generate caspase 8 and thereby promotes apoptosis [159]. Caspase 3 activated downstream of caspase 8 may also inhibit prosurvival signaling from integrins that are attached to matrix by cleaving kindlin-3 and paxillin [127,160].

Alterations in the ligand presentation by ECM can also regulate apoptosis. Integrin ligation by soluble, rather than intact, ligands can function as integrin antagonists and promote apoptosis rather than survival or proliferation [53]. Many of these soluble ligands can be created by matrix degradation during tissue remodeling. For example, endostatin, derived from collagen XVIII, as well as tumstatin, canstatin, and arresten, derived from collagen IV, all inhibit angiogenesis by promoting, among other things, endothelial cell apoptosis [78]. Endostatin binding to integrin $\alpha_5\beta_1$ decreases expression of prosurvival Bcl family members, leading to apoptosis via the intrinsic pathway [161]. Tumstatin induces endothelial cell apoptosis via integrin $\alpha_v\beta_3$, interfering with its ability to bind matrix-associated VEGF and inhibiting prosurvival FAK and PI3K/Akt signaling [162]. Similarly, arresten and canstatin, also fragments of collagen IV, bind integrins $\alpha_1\beta_1$ and $\alpha_V\beta_5$/$\alpha_V\beta_3$, respectively. In doing so, they inhibit prosurvival signaling downstream of FAK, decreasing levels of prosurvival Bcl family members (arresten) and inhibiting PI3K/Akt (canstatin) [162]. Furthermore, both arresten and canstatin activate caspases by increasing expression of Fas ligand, which activates the extrinsic apoptotic pathway [162]. Matrix fragments can promote apoptosis through nonintegrin receptors. For example, elastin fragments promote apoptosis in some cell types, likely through the ERC [25]. The proapoptotic activity of many matrix fragments suggests that these fragments disrupt normal prosurvival signaling through intact matrix molecule activation of their receptors.

While most intact matrix molecules promote prosurvival, antiapoptotic signaling, integrins that interact with specific ligands can still promote apoptosis in some cases. For example, the matricellular protein CCN1 binding to syndecan-4 and integrin $\alpha_6\beta_1$ induces expression of Bax, which promotes cytochrome C release from the mitochondria and subsequent caspase 9 activation, leading to fibroblast apoptosis [163]. In endothelial cells, thrombospondin interaction with CD36 leads to the activation of the Src kinase Fyn, inducing downstream activation of caspases 8 and 9, the former through increased expression of death domain receptors and their ligands [164]. As such, matrix fragments and specific matrix molecules may cooperate to induce apoptosis.

Relevance for tissue engineering

Designing tissue and organ replacements that closely simulate normal physiology is a challenging endeavor. One avenue to achieve this goal is to study how tissues and organs arise during embryogenesis and during normal processes of repair, and how those functions are maintained. When developing tissue replacements, one needs to consider how to avoid a strong immune response, provide the appropriate substrate for cell survival and differentiation, and provide the microenvironment needed to promote regeneration and tissue maintenance. Each of these considerations is discussed in more detail later.

Avoiding a strong immune response that can cause chronic inflammation and/or rejection

One way to avoid an immune response would involve using autologous cells, though it is time-consuming and labor-intensive to collect and expand autologous cells for use in engineered tissues, decreasing the practicality of this approach. Using autologous cells may also be detrimental in preparing engineered tissues if these cells rarely or never divide. In the case of nondividing or terminally differentiated cells, it may be beneficial to instead transplant stem cells, such as adult stem cells or induced pluripotent stem cells (iPSC) capable of differentiating into the needed cell type(s), or transplant cells differentiated from stem cells [165]. Adult stem cells, such as mesenchymal stem cells (MSC), generally do not induce an immune response after transplantation. Although they are limited in the types of cells that they can become, these cells can promote tissue repair and regeneration [166]. Unlike adult stem cells, iPSC can differentiate into every cell type and can be modified using gene editing techniques to correct any mutations that might be present in these cells [167]. Because iPSC are derived from the patient's own cells, they should not induce immune responses, at least in theory [165]. However, transplanted iPSCs and cells differentiated from them have induced immune responses, the mechanisms involved in responses against autologous cells remain unclear, and may depend upon their ex vivo manipulations prior to transplant [167]. Other drawbacks to the use of iPSC include the time-consuming nature of their preparation and subsequent differentiation and the risk of tumorigenesis from implanting undifferentiated cells [165]. Another potential strategy that avoids using cells altogether would involve the inclusion of exosomes derived from adult stem cells, such as MSC, in the engineered tissue. Exosomes are extracellular vesicles that can contain mRNA coding for specific proteins and/or the proteins themselves, including matrix-remodeling enzymes such as MMPs and ADAMs, matrix receptors such as CD44, and molecules involved in signal transduction, which can modify the matrix and matrix-mediated signaling events to alter gene expression in nearby cells [168]. Because one of the chief benefits of MSC is thought to involve their ability to modify the microenvironment, many of the benefits of MSC could be provided by MSC-derived exosomes [169]. However, exosomes still contain MHC molecules, making allogeneic exosomes susceptible to immune rejection, giving them some of the same limitations as allogeneic cells [170].

Creating the proper substrate for cell survival and differentiation

Cells transplanted directly into an injured tissue are insufficient to promote tissue regeneration, because cells need to interact with supportive ECM molecules and growth factors to survive and carry out their intended functions after transplantation [171]. To provide this supportive matrix in an engineered tissue, expanded adult cells, adult stem cells, iPSC, or heterologous cells in conjunction with immunosuppressant drugs are seeded within biocompatible implants composed of ECM molecules. Addition of growth and differentiation factors to these matrices as well as agonists or antagonists that favor cell–ECM interactions may increase the rate of successful tissue replacement. However, growth factor activity in the engineered tissue is dependent upon its stability and ability to diffuse both within the engineered tissue and in peripheral host tissue. For example, many growth factors have short half-lives in normal tissues, so it is important to increase their stability and activity to prolong their function in engineered tissues [169]. Preventing a specific type of VEGF proteolysis can increase its half-life and activity, which could be particularly important in treatment of chronic wounds, whose excessive protease levels would otherwise cleave wild-type VEGF and limit its activity. Indeed, in a mouse model of impaired healing, VEGF lacking specific proteolytic sites promoted VEGF duration and activity, leading to improved angiogenesis and healing [172]. Some of the methods used to increase the duration and activity of growth factors involve adding components of the normal ECM that stabilize these factors and/or promote their activity to the engineered tissue, covalently coupling the growth factors to a matrix molecule or biomaterial, or generating growth factors more resistant to proteolytic digestion [171]. In the case of VEGF, covalent linkage to a fibrin matrix or addition of heparin, fibronectin, and/or fibronectin fragments to the matrix promotes angiogenesis to a greater extent than addition of VEGF alone [171,173]. In addition to restricting VEGF diffusion, heparin and fibronectin fragments likely promote VEGF binding and activation of VEGFR2 [50]. In the case of fibronectin, this matrix molecule binds both VEGF and integrin $\alpha_5\beta_1$ to facilitate VEGF-induced

angiogenesis [98,100]. Based upon this example of VEGF and angiogenesis, it is clear that inclusion of matrix molecules and/or matrix fragments to an engineered tissue can promote growth factor retention and signaling in the tissue itself.

Perhaps one of the most effective substrates to use for engineering a specific tissue would be the mixture of matrix components in an acellular version of that tissue, the dermis, or tendon tissue with the cells removed, for example. Removal of the cells would decrease the likelihood of an immune response against the tissue while preserving the unique anatomy and collection of adhesive molecules that promote cell survival, proliferation, and differentiation within that tissue [174]. These acellular matrices could either provide support for endogenous cells moving into the tissue or for cells seeded within this acellular matrix. There is a limited supply of human tissues for transplant, largely from cadavers, but decellularized matrices from pigs and cows have also been used in wound therapy with positive outcomes [174]. Beyond acellular matrices, there are a number of naturally occurring matrix molecules and polysaccharides that have been used in the generation of engineered tissues. For example, fibrin, collagen, gelatin (denatured gelatin), and hyaluronan all have binding sites for multiple cell adhesion receptors that can promote cell survival, proliferation, and migration to cells within the engineered tissue and those that are recruited to the tissue [174]. These matrices can be combined with or linked to growth factors to regulate growth factor release over time. For example, growth factors placed within fibrin hydrogels can be released by fibrin proteolysis to allow growth factor delivery over time [175]. In another example, heparin-modified hyaluronan allows growth factor attachment to the associated heparin molecules, which could facilitate receptor binding and downstream signaling of some growth factors (e.g., VEGF), while retaining and sequestering other growth factors to prevent receptor binding until proteolytic release from heparin (e.g., HB-EGF) [9,176].

While the foregoing examples show that ECM molecules can be used successfully in tissue engineering, the use of natural ECM molecules and decellularized matrices in engineered tissue have several disadvantages, including the possibility of generating an immune response, variability, possible contamination, and ease of degradation [177]. Degradation of matrix molecules can alter their receptor binding, adhesive function, and downstream signaling. For example, binding of soluble, rather than intact, matrix molecule to integrins can promote apoptosis rather than cell survival, and low molecular weight fragments of hyaluronan bind to TLRs and induce inflammation [21,23,53,78,161]. Generally speaking, apoptosis and inflammation are not optimal for the success of an engineered tissue.

Likewise, artificial biocompatible materials have drawbacks in that, unlike ECM molecules, they are generally incapable of transmitting growth and differentiation cues to cells. Much attention is now devoted to the design of "semisynthetic biomaterials" in which functional regions of ECM molecules, including those that interact with receptors or growth factors or those that are cleaved by proteases, are incorporated into artificial biomaterials to impart additional functionality [171]. The inclusion of ECM-like cell-binding sites that promote cell adhesion, growth, and/or differentiation into such biomaterials may be critical in developing and maintaining functional engineered tissues by providing the appropriate cellular microenvironment. For example, because many integrins bind to the RGD motif on matrix molecules, RGD peptides can be used to make synthetic implants more biocompatible and to allow the development of tissue structure, or as antagonists to prevent or moderate unwanted cell−ECM interactions [171]. Similarly, collagen, fibrin, and vitronectin have been used to coat synthetic biomaterials to increase their biocompatibility and promote successful biological interactions [165,175,178]. However, the use of these biomaterials in engineered tissues requires additional knowledge regarding the types of cell−ECM interactions that result in the desired cellular effects within the context of the tissue of interest.

Providing the appropriate environmental conditions for tissue maintenance

To maintain tissue homeostasis, it is crucial to create a balanced environment with the appropriate cues for preservation of specific cell function(s). It is important to realize that such stasis on the level of a tissue is achieved via tissue remodeling—the dynamic equilibrium between cells and their environment. The same ECM molecule may have multiple cellular effects whose cellular outcome likely depends upon the combination of variables, such as the domain of the molecule involved in the cellular interactions, the receptor used for these interactions, and the cellular microenvironment. These variables can, in turn, be influenced by matrix remodeling, as enzymatic degradation of the ECM can release functional fragments of ECM that then alter cell−ECM interactions by removing certain binding sites while exposing others. Another important consideration is the role of mechanical tension on matrix signaling, and, depending upon the desired effect, it may be necessary to control the stiffness and pore size of the biomaterial. Hyaluronan and collagen hydrogels can be "tunable" in terms of stiffness and pore size, depending upon the concentrations of molecule, polymerization conditions, and chemical cross-linking [171,179]. One biocompatible material that is "tunable"

in terms of stiffness and pore size is starPEG, which can use heparin as a functional cross-linker. Altering the concentration or molecular weight of the starPEG changes its stiffness and pore size and can thus change the mechanical tension exerted on cells, while the use of heparin as a cross-linker yields additional functionality due to its ability to bind growth factors and enhance their signaling [171]. Combinations of this hybrid biomaterial with RGD and growth factors increased endothelial cell survival and tube formation, suggesting the potential of this approach in designing engineered tissues [180]. Interestingly, the heparin of starPEG is also able to sequester chemokines and reduce inflammation and promote healing in db/db mice, which could be particularly useful when engineering tissues for use in environments that need to reduce inflammation and promote regeneration and repair [181].

Because organ transplantation is one of the least cost-effective therapies and is not always available, tissue engineering offers hope for more consistent and rapid treatment of those in need of body part replacement, and therefore has greater potential to improve patient quality of life. The selected examples presented before illustrate that further advances in tissue engineering require additional knowledge of the basic mechanisms of cell function and of the ways they interact with the environment. The recent surge in research on ECM molecules themselves and their interactions with particular cells and cell-surface receptors has led to realization that these interactions are many and complex, allow the modulation of fundamental events during development and wound repair, and are crucial for the maintenance of the differentiated phenotype and tissue homeostasis. As such, the manipulation of specific cell–ECM interactions has the potential to modulate particular cellular functions and processes in order to maximize the effectiveness of engineered tissues.

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Chapter 7

Matrix molecules and their ligands

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Introduction

Tissue development and homeostasis requires cell−cell and cell−extracellular matrix (ECM) interactions. The ECM is not merely a structural scaffold for cell attachment but also directly presents biochemical and mechanical cues to cells and sequesters growth factors and other soluble cytokines to regulate cell signaling. Mechanical and biochemical cues elicited by the ECM permit the coordinated modulation of a wide spectrum of biological processes.

The broad array of ECM functions can be attributed to the extensive diversity of its constituent matrix molecules and their organizational complexity. For example, the three major ECM molecular categories, including collagens and elastic fibers, glycoproteins, and proteoglycans/glycosaminoglycans, are comprised of macromolecules that contain a plethora of structural motifs that are posttranslationally modified to dictate their function. The multidomain nature of ECM proteins plays a key role in their ability to incorporate many disparate biological activities within a single protein. ECM proteins are comprised of multiple domains, with complex posttranslational modifications that regulate their size and function. Moreover, ECM proteins are assembled as multicomponent polymers, and these multiprotein polymers also interact with other matrix proteins to generate an extensive interconnected, highly organized, insoluble protein network within tissues [1].

The ECM has been subcategorized as basement membrane and interstitial matrix based upon its geographical region within the tissue. The basement membrane is the thin ECM structure that separates epi-, meso-, and endothelial cell layers that anchors these cell layers to the underlying connective tissue. For example, basement membrane encases the endothelium of blood vessels and surrounds the epithelium of ductal tissues. The interstitial matrix fills the interstitial space between cells and is highly variable between tissue types.

Specialized tissue-resident cells orchestrate ECM assembly through the synthesis, secretion, organization, posttranslational glycosylation and cross-linking, and enzymatic degradation of ECM proteins and glycosaminoglycans (GAGs). Many ECM proteins require posttranslational proteolytic processing or chemical modification following their synthesis and/or secretion into the extracellular space prior to their incorporation into either the basement membrane or interstitial ECM. These modifications are critical for regulating matrix architecture, including fiber thickness and alignment, porosity, and hydration, and exert profound effects on the phenotype of the cellular constituents within the tissue. The relative abundance of different ECM components in combination with extracellular processing controls the phenotype of the resultant ECM. Accordingly, the biochemical and mechanical properties of the ECM are intimately linked. ECM synthesis and posttranslational modifications and assembly are tightly regulated processes. In this way, each tissue type is able to finely tune their ECM to match their differentiation and function and to orchestrate changes in the ECM that are necessary to maintain tissue homeostasis.

Not surprisingly, the complexity of native ECM has proven difficult to recapitulate in vitro. ECM components reconstituted into hydrogels do not possess all the properties and intricacies of the cell-generated in vivo matrix [2]. Clarifying the molecular intricacy of ECM assembly and structure/function are needed to provide critical insight to inform design principles for improved tissue engineering.

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Collagens

Collagens are key ECM constituents in most mammalian tissues. As such, they are the most abundant proteins in mammals, making up nearly 30% of total protein mass [3]. There are at least 44 distinct α chains in humans that assemble into 28 recognized types of collagen, which gives rise to significant diversity within the collagen superfamily (Table 7.1). Collagen molecules exist as homo- or heterotrimers of three type-specific collagen α chains. The repeating amino acid sequence Gly-X-Y (X and Y can be any amino acid, but Y is often proline) in a

TABLE 7.1 Types of collagen.

Type	Genes encoding α chains	Molecule	Class
I	*COL1A1* and *COL1A2*	$[\alpha1(I)]_2$, α2(I) or $\alpha1(I)]_3$	Fibrillar
II	*COL2A1*	$[\alpha1(II)]_3$	Fibrillar
III	*COL3A1*	$[\alpha1(III)]_3$	Fibrillar
IV	*COL4A1, COL4A2, COL4A3, COL4A4, COL4A5*, and *COL4A6*	$[\alpha1(IV)]_2$, α2(IV) or α3(IV), α4(IV), α5(IV) or $[\alpha5(IV)]_2$, α6(IV)	Network-forming/ basement membrane
V	*COL5A1, COL5A2, COL5A3*, and *COL5A4*	$[\alpha1(V)]_2$, α2(V) or [α1(V)]3 or $[\alpha1(V)]_2$, α4(V) or α1(V), α2(V), α3(V) or α1(XI), α1(V), α3(XI)	Fibrillar
VI	*COL6A1, COL6A2, COL6A3, COL6A5*[a], and *COL6A6*	α1(VI), α2(VI), α3(VI)	Beaded filaments
VII	*COL7A1*	$[\alpha1(VII)]_3$	Anchoring fibrils
VIII	*COL8A1* and *COL8A2*	$[\alpha1(VIII)]_2$, α2(VIII) or α1(VIII), $[\alpha2(VIII)]_2$ or $[\alpha1(VIII)]_3$	Network-forming
IX	*COL9A1, COL9A2*, and *COL9A3*	α1(IX), α2(IX), α3(IX)	FACIT
X	*COL10A1*	$[\alpha1(X)]_3$	Network-forming
XI	*COL11A1, COL11A2*, and *COL11A3*	α1(XI), α2(XI), α3(XI) or α1(XI), α1(V), α3(XI)	Fibrillar
XII	*COL12A1*	$[\alpha1(XII)]_3$	FACIT
XIII	*COL13A1*	$[\alpha1(XIII)]_3$	MACIT
XIV	*COL14A1*	$[\alpha1(XIV)]_3$	FACIT
XV	*COL15A1*	$[\alpha1(XV)]_3$	Multiplexin
XVI	*COL16A1*	$[\alpha1(XVI)]_3$	FACIT
XVII	*COL17A1*	$[\alpha1(XVII)]_3$	MACIT
XVIII	*COL18A1*	$[\alpha1(XVIII)]_3$	Multiplexin
XIX	*COL19A1*	$[\alpha1(XIX)]_3$	FACIT
XX	*COL20A1*	$[\alpha1(XX)]_3$	FACIT
XXI	*COL21A1*	$[\alpha1(XXI)]_3$	FACIT
XXII	*COL22A1*	$[\alpha1(XXII)]_3$	FACIT
XXIII	*COL23A1*	$[\alpha1(XXIII)]_3$	MACIT
XXIV	*COL24A1*	$[\alpha1(XXIV)]_3$	Fibrillar
XXV	*COL25A1*	$[\alpha1(XXV)]_3$	Membrane-bound, brain-specific
XXVI	*COL26A1*	$[\alpha1(XXVI)]_3$	Other
XXVII	*COL27A1*	$[\alpha1(XXVII)]_3$	Fibrillar
XXVIII	*COL28A1*	$[\alpha1(XXVIII)]_3$	Other

FACIT, Fibril-associated collagens with interrupted triple helices; *MACIT*, membrane-associated collagens with interrupted triple helices.
[a]*COL6A5 has also been designated COL29A1.*

portion of each α chain enables folding of a tight, right-handed triple helix domain, the signature structural motif of the collagens. Additional domains within the collagen molecule determine the different supramolecular assemblies formed after secretion into the extracellular space, which are used to classify collagens into the subfamilies discussed later [3].

Fibrillar collagens

Collagens that assemble to form long, rod-like bundles called fibrils are classified as the fibrillar collagens. Fibrillar collagens account for a large majority of the collagen content in humans and are comprised of collagen I, II, III, V, XI, XXIV, and XXVII, with collagens I–III as the most common [4]. Most collagen fibrils are heterotypic, containing multiple types of collagen within a single fibril. For example, collagen fibrils in the skin are often composed of collagen I and III, and cartilage is typically collagen II/IX/XI or collagen II/III. Collagen fibrils typically contain one long triple helical region and assemble into a fibrillar suprastructure that enables them to provide structural support to tissues and bear large tensile loads. Due to their mechanical strength, collagen fibrils are typically found in stromal ECM and connective tissue such as skin, tendons, ligaments, and bone.

Fibrillar collagen biosynthesis has been the most extensively studied and is illustrated in Fig. 7.1. Fibrillar collagen α chains are synthesized in the endoplasmic reticulum as pro-α chains, with long propeptide regions on either side of the triple helical domain. Prior to triple helix formation, the α chains can undergo extensive post-translational modifications, including proline and lysine hydroxylation, lysine and hydroxylysine glycosylation, and tyrosine sulfation [3,5]. Proline hydroxylation by prolyl hydroxylases is particularly critical for the stability of the ensuing triple helix. Associations between pro-α chains begin with disulfide bonding among their C-terminal propeptides and initiate the formation of the triple helix near the C-terminus [6,7]. Triple helix formation then propagates toward the N-terminus like a zipper forming a procollagen molecule. Formation of the complete triple helix quenches further posttranslational modification of the triple helical region. The procollagen molecule is shuttled to the Golgi, where it is packaged for secretion into the extracellular space.

Following their extracellular secretion, extracellular BMP1/tolloid-like proteinases and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family proteinases cleave the C- and N-terminal propeptides, respectively, yielding a mature collagen molecule. Mature collagen molecules undergo entropy-driven self-assembly into small microfibrils of about 20 nm diameter and 4–12 μm in length. The collagen molecules pack together in a longitudinally staggered pattern that forms areas of high and low packing density. This causes the characteristic 67 nm periodic banding pattern observed in collagen fibrils. Immature microfibrils associate with each other and are organized into very long mature collagen fibrils that can be 20–500 nm in length [8].

The axial and radial growth of fibrils is controlled by several different molecules. Collagens V and XI play a

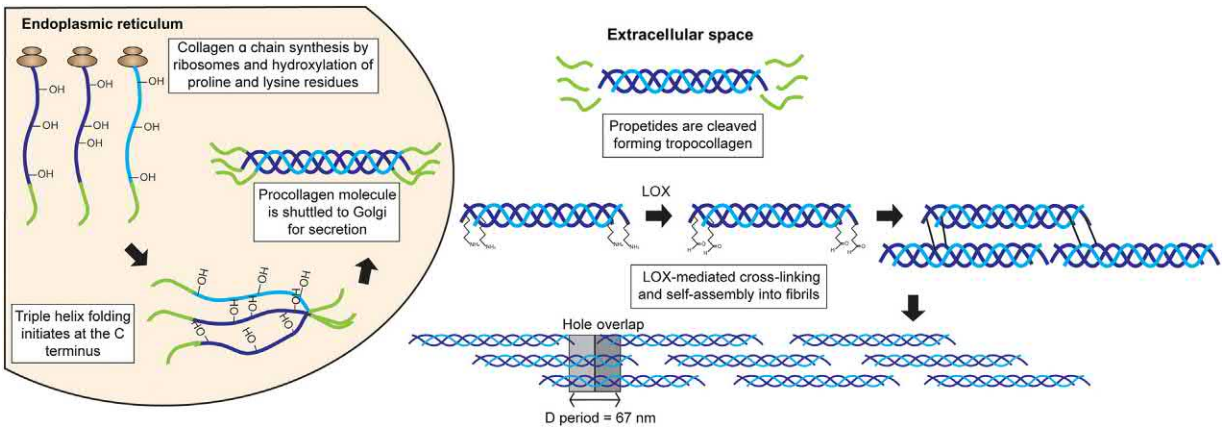

FIGURE 7.1 Collagen synthesis and fibrillogenesis.
Collagen α chains are synthesized by ribosomes and are modified by prolyl and lysyl hydroxylases in the endoplasmic reticulum. Proline hydroxylation within the helical region is required for helix stability. Lysine hydroxylation in the telopeptide region by lysyl hydroxylase 2b influences the structure of future LOX-mediated cross-links. Three α chains (depicted are the two α1 chains and one α2 chain of collagen I) associate near the C-terminus and triple helix folding propagates toward the N-terminus. The resulting procollagen molecule is transported to the Golgi and secreted into the extracellular space, where the C- and N-terminal propeptides are enzymatically cleaved. Collagen molecules self-assemble into microfibrils during which LOX catalyzes the formation of reactive aldehydes in telopeptide lysine and hydroxylysine residues that spontaneously react to form intermolecular cross-links. The packing pattern of collagen molecules within a microfibril yields its characteristic banding pattern that can be visualized by electron microscopy or atomic force microscopy. *LOX*, Lysyl oxidase.

key regulatory role in fibril assembly. Much of the N-terminal propeptide remains uncleaved in these two collagen types, which may sterically hinder incorporation of additional collagen molecules. Thus fibril size is related to the ratio of the major fibril component (type I or II) to the minor component (type V or XI), with a high V/XI content yielding thinner fibril bundles and a low V/XI content yielding thick fibrils [9]. Healthy collagen I fibrils in skin, tendon, ligaments, and bone contain 5%–10% collagen V, and collagen II fibrils in cartilage contain 5%–10% collagen XI. In addition to collagens V and XI, in vivo manipulations have revealed that collagen-binding small leucine-rich repeat proteoglycans (SLRPs) such as biglycan, decorin, lumican, and fibromodulin facilitate both fibril elongation and lateral association with other growing collagen fibrils [8]. The FACIT (fibril-associated collagens with interrupted triple helices) family of collagens also plays a critical role in fibrillar collagen structure and is discussed extensively later.

In the final step of collagen maturation, fibrils undergo varying degrees of enzymatic and nonenzymatic covalent cross-linking. The mechanical properties of fibrillar collagens are highly dependent on these modifications. The lysyl oxidase (LOX) family enzymes catalyze the oxidative deamination of peptidyl lysine and hydroxylysine residues in the telopeptide region adjacent to the triple helix in the collagen molecule. The aldehyde produced by this reaction spontaneously reacts with another LOX-derived aldehyde or with the amine group in helical lysine or hydroxylysine residues to form intra- and intermolecular cross-links. These initial divalent cross-links can mature further into multivalent cross-links through the incorporation of additional amino acids [10]. The hydroxylation of telopeptide lysines by lysyl hydroxylase 2 during collagen synthesis in the endoplasmic reticulum gives rise to cross-linking maturation products with distinct mechanical properties and are enriched in bone, ligaments, tendons, and pathologically fibrotic tissues [10,11]. Collagen in softer connective tissues such as skin or cornea contains lower levels of hydroxylated telopeptide lysine residues and therefore contains a much different profile of mature collagen cross-links. Tissues finely tune the quantity and quality of LOX-mediated collagen cross-links to meet their structural needs. Although many of the cross-linking maturation products have been identified, the precise mechanisms by which they all form and are regulated are not fully understood. Recent advances in mass spectrometry methods have allowed quantification of specific LOX-mediated cross-linking products in tissues to understand their role in tissue structure and in the progression of fibrotic disease [12,13]. LOX activity has traditionally been difficult to assay in situ because the posttranslational modifications and copper cofactor required for catalysis decouple extracellular LOX levels from its enzymatic activity. A novel imaging approach seeks to overcome this challenge using gadolinium-based sensors that detect the oxidized lysine residues in collagen that form after LOX modification [14]. This is a promising approach to noninvasively monitor the progression of fibrotic diseases and therapeutic response.

Collagen cross-linking can also be mediated via transglutaminase 2, which links collagen and fibronectin, as well as by advanced glycation end products, which are generated spontaneously as the collagen ages in tissue.

Fibril-associated collagens with interrupted triple helices (FACIT)

Collagens type IX, XII, XIV, XVI, XIX, XX, XXI, and XXII comprise the FACIT subfamily. As their name implies, these collagens contain at least two triple helical domains and associate with the surface of collagen fibrils, despite not forming fibrils themselves. Many FACIT collagens also share homologous domains with other noncollagenous ECM proteins such as fibronectin, thrombospondin-1, and von Willebrand factor. The FACIT subfamily is a more heterogenous and structurally complex group of collagens relative to the fibrillar collagens, which may be due to their roles in facilitating interactions between specific fibril types and unique tissue environments [15].

Collagen IX is the best characterized FACIT collagen and is found covalently bound to collagen II fibrils in cartilage. Collagen IX links collagen fibrils to other collagen fibrils, and collagen fibrils to ECM components, as well as collagen fibrils to cells [16]. Consistent with these functions, mutations in any of the three collagen IX α chains cause multiple epiphyseal dysplasia, a rare disorder characterized by defects in cartilage mineralization at the ends of growing bones. Compromised cartilage mineralization leads to early onset osteoarthritis and short stature and limbs.

Collagens XII and XIV are both homotrimers and associate with collagen I fibrils in connective tissues. Collagen XII helps to organize and stabilize type I collagen fibrils to maintain muscle and bone integrity. One of collagen XII's noncollagenous domains (NC3) interacts with other matrix proteins including decorin, fibromodulin, and tenascin. The NC3 domain of collagen XII can be alternatively spliced to form a smaller, minor isoform, collagen XIIb, which lacks this binding activity and resembles collagen XIV. Type XII collagen levels are elevated in fibrosis and in the desmoplastic stroma of some tumors, implying that collagen XII facilitates the aberrant organization of the interstitial ECM observed in these conditions [17,18]. However, a mechanistic understanding of these observations is not known and is an active area

of investigation. Collagen XIV regulates radial fibril growth and the integration of fibrils into fibers in collagen-dense structural tissues such as tendon [19]. It is found in skin, tendon, cornea, and articular cartilage, particularly in the area of high mechanical stress, which suggests a role for collagen XIV in regulating fibril mechanical properties [20]. Consistently, the skin of mice bearing a *Col14a1* knockout is mechanically inferior and has reduced maximum tensile loading. Developing tendons in *Col14a1*-null mice also displayed disrupted fibrillar architecture and defects in load bearing, which were not observed in mature *Col14a1*-null tendons [19]. This suggests a temporal and tissue-specific role of type XIV collagen in fibrillogenesis.

The remaining FACIT collagens have not been as well characterized. Most are tissue-restricted and are believed to play similar roles related to organizing fibrillar collagen structures and as substrates for cell adhesion [21]. A small subset such as collagen XXII can also localize in basement membrane [22].

Basement membrane–associated collagens

Collagen IV is the major collagenous component of basement membranes. Six individual genes code for collagen IV α chains, producing at least three distinct, tissue-specific heterotrimeric collagen IV molecules [23]. The trimeric molecules contain three domains: an N-terminal 7S domain, the triple helical domain, and a C-terminal NC1 domain. Four molecules come together and associate via their 7S domains, and the NC1 domain at the opposite end binds the NC1 domain of another molecular in a collagen IV tetramer [24]. Continuous NC1 end-to-end linking of tetramers along with lateral associations of molecules via their triple helical domains form the collagen IV network suprastructure [25].

Other network-forming collagens include the short-chain collagens VIII and X. These molecules share a high degree of sequence homology and form hexagonal networks. Collagen X is produced by hypertrophic chondrocytes and can only be found within the hypertrophic zone of the epiphyseal growth plate cartilage. On the other hand, collagen VIII can be found in the endothelial basement membranes of several tissues [26]. Collagen VIII is a major component in the Descemet membrane in the cornea, and mutations can cause corneal clouding [27,28]. Furthermore, collagen VIII has been shown to participate in angiogenesis, tissue remodeling, and fibrosis, and may also play a role in stabilizing atherosclerotic plaques [29].

Other collagens

Collagen VII forms anchoring fibrils that are critical for linking basement membrane to the interstitial matrix. Two collagen VII molecules join together near their C-terminal to form a U-shaped dimer, which then traps interstitial fibrillar collagens I, III, and V. The NC1 domains at the N-terminals of each molecule bind basement membrane proteins collagen IV, laminin-332, and laminin-311 with high affinity, thus stabilizing the interstitial matrix with the basement membrane [30]. Mutations in COL7A1 lead to dystrophic epidermolysis bullosa, a disorder causing blistering and scarring of the skin [31]. Circulating autoantibodies to collagen VII are also known to cause a form of epidermolysis bullosa in addition to being linked with inflammatory bowel disease and Crohn's disease [32,33].

The MACIT (membrane-associated collagens with interrupted triple helices) collagens are a subfamily of transmembrane collagens (XIII, XVII, XXIII, and XXV) that anchor cells to the basement membrane. Collagen XVII is found in hemidesmosomes and is important for epithelial adhesion to the basement membrane, particularly in areas of high mechanical stress [34].

Collagens XV and XVIII constitute the multiplexins (*multiple* triple heli*x* domains and *in*terruptions), which are a subfamily of basement membrane–associated collagens. Both of these collagens share structural homology and are proteoglycans. Collagen XV contains a variable glycosaminoglycan side chain comprised mostly of chondroitin sulfate, whereas collagen XVIII contains several heparan sulfate side chains. Mutations in collagen XVIII cause Knobloch syndrome, which leads to degeneration of the retina and vitreous, causing extreme nearsightedness and often, blindness [35]. Proteolytic cleavage of collagen XVIII can release a protein fragment called endostatin, an antiangiogenic heparin-binding peptide. Endostatin inhibits angiogenesis by reducing migration and proliferation of vascular endothelial cells [35–37]. A number of other collagenous and noncollagenous ECM proteins have since been shown to release bioactive fragments upon enzymatic cleavage, some of which are also antiangiogenic. These fragments are collectively known as matricryptins.

Major adhesive glycoproteins

Fibronectin

The glycoprotein fibronectin is a prominent and ubiquitous ECM constituent that plays a critical role in cell adhesion, migration, proliferation, and differentiation [38]. Cell binding to fibronectin modifies cell behavior that has endowed fibronectin with key features that mediate important biological processes such as wound healing and embryonic development and implicates the molecules in pathological processes including fibrosis and cancer. Fibronectin exists as a dimer of two 230–250 kDa subunits, each composed of three types of repeating modules:

FN1, FN2, and FN3 (Fig. 7.2). Interestingly, these fibronectin modules are also commonly found within domains of many other ECM and ECM-associated proteins. Cellular fibronectin is secreted by interstitial fibroblasts and other stromal cells that also act to organize fibronectin into an insoluble fibrillar network. Hepatocytes in the liver also secrete soluble fibronectin that circulates in blood plasma with a concentration near 300 μg/mL.

The fibronectin dimer forms from two nearly identical fibronectin monomers joined by a disulfide linkage near the C-terminal of each subunit. Each subunit contains 12 FN1 modules, 2 FN2 modules, and 15−17 FN3 modules. Fibronectin contains nine glycosylation sites (seven N-linked and 2 O-linked) per subunit, such that the molecule is on average 5% carbohydrate. Glycosylation regulates fibronectin stability and its affinity to some of its binding partners such as collagen [39,40]. Unlike collagen, fibronectin is produced from only one gene, and alternative splicing of the mRNA transcript causes variability within the fibronectin protein. The number of FN3 modules depends on the presence of two extra domains, extra domain A (EDA) and extra domain B, located between the 11th and 12th FN3 modules and 7th and 8th modules, respectively. In addition, a variable (V) region between ^{14}FN3 and ^{15}FN3 can be spliced to form 5 distinct variants bringing the total number of human fibronectin isoforms to 20 [41].

The diverse functional domains within the fibronectin dimer give rise to its diverse biological activity. For instance, regions within the FN1 repeats mediate its binding to collagen (^{6}FN1−^{9}FN1 with ^{1}FN2−^{2}FN2) and fibrin (^{4}FN1−^{5}FN1 and ^{10}FN1−^{12}FN1) and contain the weaker of two heparin-binding domains (^{1}FN1−^{5}FN1) [42]. A third fibrin-binding site is exposed near the collagen-binding site following chymotrypsin digestion [43]. The FN3 repeats contain the strongest heparin-binding site (^{12}FN3−^{14}FN3) and most importantly, the major integrin-binding motif, RGD, within ^{10}FN3. The RGD binds integrins α3β1, α5β1, α8β1, αvβ1, αIIbβ3, αvβ3, αvβ5, and αvβ6. Importantly, ^{9}FN3 contains a DRVPPSRN synergy sequence, which exclusively binds α5β1 and αIIbβ3 integrins. Integrin binding to the synergy site enhances

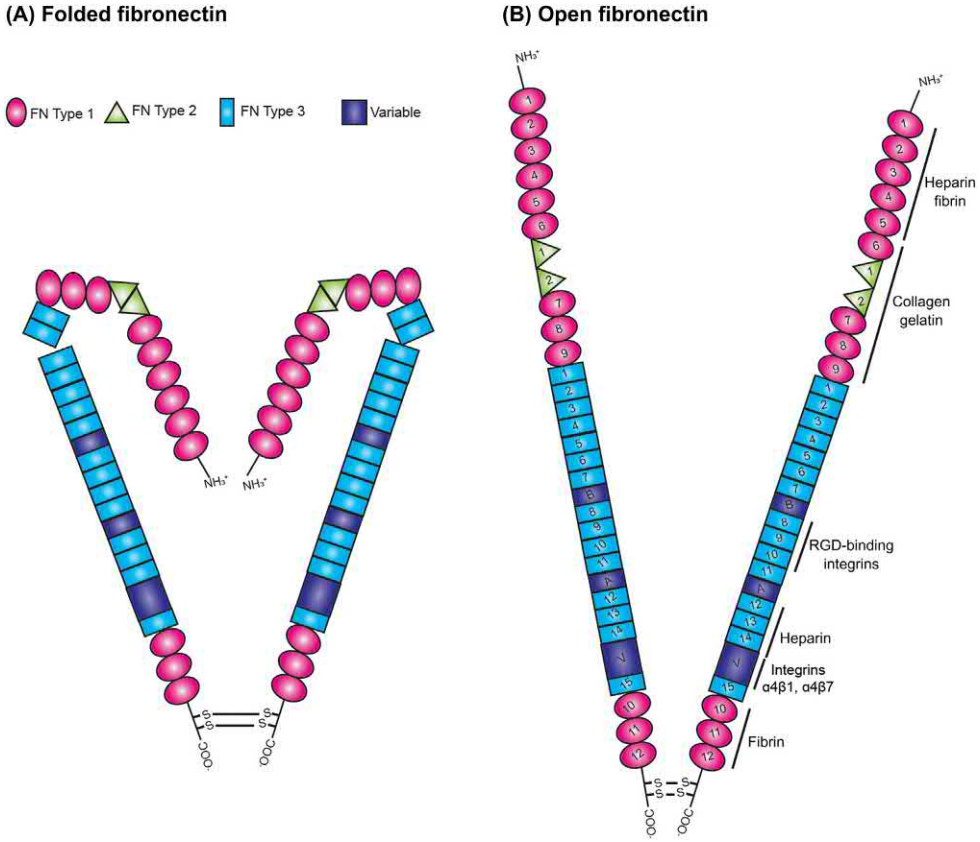

FIGURE 7.2 Fibronectin structure.
(A) Soluble fibronectin dimers adopt a folded conformation that opens upon cell binding. (B) Fibronectin contains three types of repeating domains, FN1−3, two alternatively spliced FN3 domains, FNA and FNB, and an alternatively spliced variable region. Integrin ligation of fibronectin and cell-generated tension open the fibronectin molecule exposing fibronectin-binding sites that initiates fibronectin assembly into a fibrillar matrix. Fibronectin also binds heparin, collagen, and fibrin at the sites shown.

cell spreading, fibril assembly, and can elevate cytoskeletal tension to reinforce focal adhesion signaling [44–46]. A second integrin-binding site, LDV, in the V regions is recognized by α4β1 and α4β7, and α4β1 and α9β1 integrins also recognize a site in the EDA region.

In contrast to spontaneous collagen fibrillogenesis, fibronectin's assembly into a fibrillar network is cell-mediated [38]. The first step in fibronectin fibrillogenesis is the binding of soluble fibronectin to a receptor on the cell surface. Based on cell binding and network assembly experiments with isolated fibronectin peptides and RGD mutations, the N-terminal 70 kDa region (70K; ^{1}FN1–^{9}FN1) likely contains the initial cell-binding cite that triggers subsequent fibrillar assembly [47]. The binding of soluble fibronectin by a cell force induces a conformational change in the fibronectin that exposes a cryptic site that can then interact with additional fibronectin molecules [48,49]. Cell-mediated tension through the integrin–fibronectin bond is predicted to facilitate continuous interaction with additional fibronectin molecules and elongation of the fibronectin fibril [50]. Cell adhesion to the RGD motif then mediates fibrillar elongation, with the synergy site also being implicated in fibronectin matrix assembly [45]. Consistently, mutations that prevent synergy site binding disrupt assembly, which can be rescued by enhancing integrin activation with Mn^{2+}. Integrin α5β1 binding of fibronectin and the ensuing signaling that activates cell contractility have also been implicated in fibronectin assembly [51].

As one of the major cell adhesive ECM proteins, fibronectin is crucial for a large number of biological processes. For instance, fibronectin plays a critical role in wound healing. Fibronectin associates with fibrin in blood clots to make up the provisional matrix, where it promotes the migration of fibroblasts, macrophages, and new epithelial cells into the wound site and facilitates the formation of a mature ECM [52]. In injuries that limit the production of endogenous fibronectin, such as following irradiation, topically applied fibronectin can significantly improve wound healing [53]. Moreover, biomaterials engineers showed that adsorption of fibronectin onto poly(ethyl acrylate) (PEA) exposes the assembly initiation site to stimulate spontaneous organization of fibrillar fibronectin at the material surface. This strategy fortuitously enhances integrin-binding and enables optimal growth factor presentation to cells. Not surprisingly, the application of PEA materials facilitates bone regeneration by stimulating osteoblast differentiation at remarkably low concentrations of BMP-2 [54].

Fibronectin also plays essential roles in embryonic development, and fibronectin-null mutations are embryonic lethal at day 8.5 [55]. In addition, fibronectin is especially abundant in many solid tumor types, where it potentiates protumor integrin signaling and its deposition at secondary sites helps facilitate tumor metastasis [46].

Laminin

Along with collagen IV, laminins are the other major constituents of basement membranes. However, laminin is the dominant adhesive protein in the basement membrane, and so it serves as a crucial link between cell layers and the basement membrane. Laminin is a large, 400–900 kDa heterotrimeric glycoprotein consisting of three polypeptide chains: α, β, and γ. There are 5 different α chains, 3 β chains, and 3 γ chains all encoded by different genes that combine to form at least 15 distinct laminin proteins [56]. Laminin nomenclature is based upon which chains compose the protein, such that laminin-511 is the laminin made up of α5, β1, and γ1 chains [57].

The main function of laminin is to provide sites for cell attachment and to assemble together with other proteins to generate a structurally functional basement membrane. Laminins self-polymerize into layered sheets and filaments that often contain multiple different laminin chains. Laminin polymerization facilitates the organization of the basement membrane, such that in the absence of laminin chains, basement membrane assembly is disrupted, despite the presence of other basement membrane components including collagen IV, perlecan, and nidogen. Critically, however, laminin does not directly bind collagen IV but is linked to the collagen scaffold via nidogen, emphasizing the key role of nidogen in the structural integrity of the basement membrane [58].

Several different cell surface receptors bind laminin including integrins α1β1, α2β1, α3β1, α6β1, α6β4, α7β1, α9β1, and αvβ3; syndecans 1 and 4; 67 kDa laminin receptor; and α-dystroglycan. The integrin-binding sites vary with the type of laminin. The main integrin-binding site is in the LG3 domain near the C-terminal end of the α chain [59]. Interestingly, recombinant LG3 domains do not bind integrin, and it is now known that a conserved glutamate residue in the γ chain is also required for integrin binding [60]. This Glu is absent in the γ3 chain; therefore laminin-113 and laminin-213 do not bind integrins [61].

Laminins play a critical role in tissue maintenance and cell survival. Along with fibronectin, collagen purified laminins are widely used in cell culture. In particular, laminin-111 is present in high concentrations in the commercially available reconstituted basement membrane hydrogel Matrigel. Dysfunctional laminins are the cause of multiple pathologies. For example, impaired function of laminin-211 and laminin-332 can cause congenital muscular dystrophy and junctional epidermolysis bullosa, respectively [62]. Laminins also play a role in tumor metastasis. Tumor cells that overexpress LAMA4 have a high metastatic efficiency, and exposure to integrin-binding epitopes of laminin in the premetastatic niche can awaken dormant cells to induce the formation of metastatic lesions [63,64].

Elastic fibers and microfibrils

While collagens resist tensile loads in tissues, elastic fibers impart tissues with the ability to stretch and recover from transient deformation. Not surprisingly, elastic fibers are abundant in tissues in which mechanical deformations are common and critical to their function, such as lungs, arteries, and skin. Elastic fibers exhibit extraordinary elasticity and undergo years of strain/recoil cycles without losing their mechanical properties [65]. By way of comparison, the extensibility of elastic fibers is five times greater than that of a rubber band of the same cross-sectional area. Elastic fibers are also acknowledged as providing important biochemical cues for cells such that recent advances in tissue engineering and regeneration have started utilizing these ECM fibers to improve results [48].

The highly elastic nature of elastin fibers is imparted by the structure of their main component, elastin. Elastin forms from its soluble precursor, tropoelastin, which self-associates immediately after synthesis into elastin polymers. The structure of tropoelastin monomers was only recently elucidated. It was shown that interspersed hydrophobic domains form large coils such that the shape is dominated by a condensed coil that confers elastic behavior at the level of the monomer [66]. Tropoelastin also has a separate cell-interacting domain outside the coiled structure. In a similar manner to collagen, lysine side chains of elastin can also be oxidized by the LOX family of enzymes to form reactive aldehydes that mediate cross-linking of elastin fibers and regulate elastin network architecture.

Elastic fibers consist of both elastin and other associated microfibrillar proteins such as fibrillin. Fibrillins serve as a scaffold to organize elastic fibers and are found surrounding the surface of amorphous elastin [67]. Fibrillins are also found as microfibrils in the ECM of many tissues independent of elastin. Fibrillins are important for the integrity and stability of elastic fibers and some nonelastic ECM. Not surprisingly, mutations in fibrillin-1 cause Marfan syndrome, a disease affecting the connective tissue [68]. Marfan syndrome can present with a multitude of symptoms, but the most common and serious consequences of the Marfan-associated fibrillin-1 mutations are cardiovascular, skeletal complications, and visual defects. Fibrillin mutations are also associated with a dysregulation of TGF-β activity [69]. Additional proteins associated with elastic fibers and microfibrils include fibulins, elastin microfibril interfacers, and microfibril-associated glycoproteins [70,71]. The importance of the cooperation between elastin and microfibrils in tissue function is highlighted by studies in which germline deletion of either elastin, fibrillin-1, or fibulin-1 induces mouse mortality shortly after birth.

Other adhesive glycoproteins and multifunctional matricellular proteins

Vitronectin

Vitronectin exists in the ECM of numerous tissues and circulates at high concentrations in blood. Cell adhesion to vitronectin is primarily mediated via integrin recognition of an RGD site in the molecule. Interestingly, although many integrins interact with vitronectin, one of the main fibronectin-binding integrins, $\alpha5\beta1$, cannot bind to vitronectin. Vitronectin can bind other matricellular proteins as well, including collagen, thrombin, heparin, plasminogen, and plasminogen activator-1. Vitronectin plays a role in wound healing, viral infection, and tumor growth [72,73]. However and somewhat surprisingly, the vitronectin knockout mouse apparently develops normally, and the adult mice do not exhibit any obvious pathological defects, suggesting this ECM molecule is not essential and/or that other proteins are able to compensate for its loss [74].

Thrombospondins

Thrombospondins are a five-member family of proteins composed of repeating EGF-like domains, Ca^{2+} binding domains, and TSP1 and TSP2 contain thrombospondin type-1 repeat (TSR) domains. This family forms homotrimeric (TSP1 and TSP2) and homopentameric (TSP3, TSP4, and TSP5/COMP) structures that assemble via a coiled-coil oligomerization domain near their N-terminus. Thrombospondins are cell adhesive and bind many ECM proteins such as fibronectin, collagens, laminin, and fibrinogen [75]. TSP1 can also bind latency-associated peptide to induce the release of active TGF-β [76]. The thrombospondin family has been implicated in a diverse range of activities related to their binding functions and ability to modulate cell behaviors. One important example is the antiangiogenic function of TSP1, which inhibits endothelial cell proliferation through its TSR domain [77,78]. This antiangiogenic property forms the basis of some TSP1-based therapies in a handful of contexts including eye diseases and cancer. TSR domains can be found within a plethora of other extracellular and cell surface proteins. Notably, this includes ADAMTS family of proteases responsible for processing of procollagen and von Willebrand factor as well as ECM remodeling through cleavage of lecticans [79].

Tenascins

The tenascin family includes four members, tenascins C, R, W, and X, that share structural similarities and

modulate cell adhesion and cellular biochemical signaling. The tenascins form rod-like structures containing EGF-like domains and fibronectin type 3 repeats. Tenascins assemble into hexamers via interactions between their oligomerization domains in the N-terminus. Tenascin expression is often limited and tissue-restricted with tenascin R only found in the nervous system, tenascin X primarily located in muscle connective tissue, and tenascin W in kidney and bone [80]. Tenascin C can be found in connective tissues and the nervous system but is also expressed at the site of tissue injury and during development and is often overexpressed in some cancers [81]. The effects of tenascin C are pleiotropic and context-dependent. Tenascin C can bind multiple integrins, although typically the tenascin-integrin adhesions are weak and do not stimulate cytoskeletal rearrangements consistent with focal adhesion assembly and cellular tension generation [82,83]. Interactions between tenascin C and ^{13}FN3 within fibronectin can induce cell-adhesion detachment from fibronectin to reduce integrin adhesion signaling [84]. Consistently, cleavage of tenascin C by meprin can rescue cell adhesion to fibronectin [85]. Tenascin C levels are also elevated in sites of high mechanical stress, suggesting it may be involved in adaptive ECM remodeling and/or signaling.

Proteoglycans

Proteoglycans are indispensable components of the interstitial ECM. All proteoglycans consist of a core protein that has at least one, but usually multiple, serine O-linked glycosaminoglycan chains. Glycosaminoglycans are unbranched polysaccharide chains composed of repeating disaccharide units, typically an amino sugar with an uronic sugar [86]. The highly hydrophilic glycosaminoglycan chains in proteoglycans trap large amounts of water within the matrix, which endows proteoglycans with the ability to regulate tissue turgidity and implicates these molecules as modulators of tissue biomechanical properties and viscoelasticity [1]. There are four classes of glycosaminoglycans: hyaluronan, heparin/heparan sulfate, chondroitin sulfate, and keratan sulfate.

Hyaluronan and lecticans

Hyaluronan is a large, 5−20,000 kDa (3−7 MDa average) glycosaminoglycan prevalent in tissues throughout the body, especially in the brain. The hyaluronan-binding proteoglycans are versican, aggrecan, brevican, and neurocan, known collectively as the lecticans or hyalectins. The binding of the core proteins to hyaluronan is noncovalent and stabilized by a link protein (Fig. 7.3). Hyaluronan is unique among the GAGs in that it is not sulfated, and it is synthesized at the plasma membrane rather than in the Golgi. It is synthesized by one of three hyaluronan synthases that add sugars to the cytoplasmic end of the chain while it elongates in the extracellular space. Hyaluronidases in the interstitial matrix degrade hyaluronan and are responsible for maintaining the steady turnover in the ECM with a half-life spanning from hours to weeks, depending on the tissue [87,88]. There are six known cell surface receptors for hyaluronan, and the two best characterized are CD44 and receptor for hyaluronan mediated motility (RHAMM). CD44-binding regulates hyaluronan clearance and importantly, can interact with growth factor receptors such as EGFR and TBRI to influence their activity and growth factor signaling [89,90]. As its name suggests, RHAMM-binding of hyaluronan

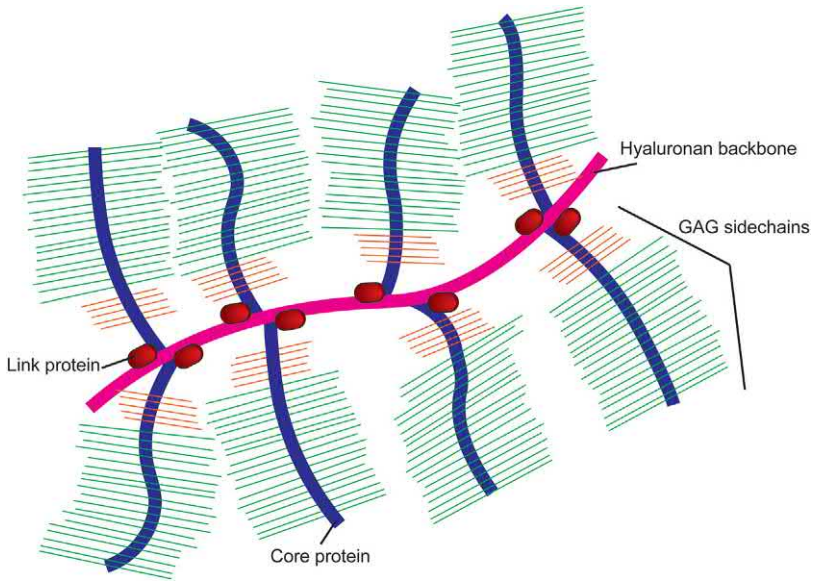

FIGURE 7.3 Hyaluronan binds proteoglycans in the ECM.
The core protein of lecticans binds numerous glycosaminoglycan chains and attaches to hyaluronan with the help of link protein. Many lecticans assemble along a hyaluronan backbone forming a massive, carbohydrate-rich ECM macromolecule. Proteoglycans regulate the hydration of tissue ECMs, which is a major determinant of overall tissue viscoelasticity and regulates interstitial pressure. *ECM*, Extracellular matrix.

modulates cell motility but also regulates cell cycle progression.

Like some other glycosaminoglycans, hyaluronan functions to resist compressive stresses. However, unlike other glycosaminoglycans, hyaluronan lowers its viscosity under sheer stress without losing its elasticity, rendering it an ideal biological lubricant. Hyaluronan is a major component of synovial fluid, which absorbs shock and lubricates joints. Hyaluronan also serves as a backbone to bind a large number of aggrecan molecules, the dominant proteoglycan in cartilage. About 90% of aggrecan mass consists of other bound glycosaminoglycans, primarily chondroitin sulfate, and this high concentration of GAGs contributes to the hydrated gel structure of cartilage that supports its large compressive loading function. Aggrecan also plays an important role in chondrocyte differentiation and facilitates chondrocyte-ECM interactions [91]. Furthermore, the gel-like phase created by hyaluronan-rich matrices can contribute to tumor pathology. Notably, high expression of hyaluronan in pancreatic tumor stroma plays a major role in generating the abnormally high interstitial pressure associated with this disease that compromises vascular integrity and impedes drug delivery, which have been attributed to the poor patient outcome associated with these cancers [92,93].

Hyaluronan with other lecticans also contribute to several other important biological processes including normal processes such as embryonic development and wound healing, as well as diseases including chronic inflammation and tumor metastasis [94–96]. The combination of hyaluronans biochemical properties in conjunction with its unique biomechanical properties endows hyaluronan with unique features that have motivated its widespread application as a facile material for tissue engineering applications [97]. Moreover, hyaluronan production can form a hydrated pericellular matrix around cells that influences the traffic of soluble factors and pericellular processes. These matrices surround gastrulating cells and are found within developing organs, associated with cells participating in wound repair, and are critical for arterial smooth muscle cell proliferation [98,99].

Perlecan

The major proteoglycan within basement membranes is the heparan sulfate proteoglycan perlecan. Perlecan is an essential ECM component, and most mice harboring a perlecan knockout exhibit embryonic lethality due to aberrant brain and skeletal development. Perlecan is particularly important in vascular basement membrane, where it helps preserve endothelial barrier function [100]. In addition to its role in basement membrane structure/function, perlecan is also present in the pericellular matrix of fibroblasts. In the interstitial ECM, heparan sulfate side chains sequester cytokines and other soluble molecules to modulate a plethora of cell and tissue behaviors critical for tissue homeostasis.

Small leucine-rich repeat proteoglycans and syndecans

Two other important proteoglycans include the SLRPs and syndecans. SLRPs are a diverse group of matricellular proteins containing leucine-rich repeat motifs that link with a variety of glycosaminoglycans. As discussed above, the SLRP decorin can modulate collagen fibrillogenesis, but the binding activity of SLRPs have also been implicated in inflammation and growth factor signaling [101]. Syndecans are a four-member group of transmembrane receptors that contain heparan sulfate and chondroitin sulfate chains. Syndecans are ECM receptors but can also facilitate cell–cell adhesion, and their extracellular GAG chains bind various growth factors.

Conclusion

Once dismissed as a biologically inert scaffold, research over the past few decades demonstrates the integral role played by the ECM in orchestrating many biological processes, including development, tissue homeostasis, wound healing, and disease. Not only do cell-ECM interactions directly modulate cell behavior, but ECM constituents also regulate the local abundance of matrix-associated signaling molecules and dictate the local and global mechanical properties of the tissue. As such, the ECM is critical for successful tissue engineering. Clarification of the role played by ECM composition, posttranslational modification, and organization and how these features direct cell differentiation and tissue-specific function will inform a more accurate design of materials to improve engineered tissues.

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Chapter 8

Morphogenesis and tissue engineering

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Introduction to tissue morphogenesis

The development of the human body from a zygote to its adult shape requires a tremendous feat of organization of cells. During this process, cells proliferate, migrate, differentiate, survive, or die in a highly controlled fashion, to eventually arrange themselves into tissues and organs (Fig. 8.1). All these controlled cellular mechanisms constitute the basis for *tissue morphogenesis*, literally defined as the *generation of tissue shape* [1,2].

Physiologically, the *shape* or *structure* of tissues is highly optimized to their biological functions. Indeed, many structures are unique to the organ in which they are found and fulfill highly specialized functions, such as kidney nephrons that filter blood and produce urine, or pancreatic islets that secrete the hormone insulin into a rich microvasculature to regulate blood glycemia. In contrast, some structures are shared between different organs, in which cases they perform similar or identical functions; for example, blood vessels bring nutrients to and remove waste products from most of the tissues, ensuring tissue viability. Because tissue structures have evolved to be functionally optimal, damage to the structures often results in partial or total loss of functions.

Nevertheless, it is the biological *functions* of tissues that are to be preserved in order to maintain life, acquire survival advantages, or perpetuate the species. To preserve biological functions throughout life, tissues undergo constant renewal and remodeling. At steady state the internal maintenance of tissue is called *homeostasis* and mainly includes cell renewal and metabolism control, yet without substantial structural changes. In addition to internal regulation, tissues adapt to external environmental variations. In these cases, tissue *remodeling* can result in structural modifications in the tissue, often to resist those environmental changes. For instance, bone structures remodel to reinforce areas where physical forces are applied [3]. In fact, biological tissues are highly *dynamic* systems.

Apart from the developmental stages, *morphogenesis* also occurs at an adult age, for example, upon wounding or in disease. Interestingly, some tissues possess natural abilities to regenerate when damaged, meaning that they can rebuild their initial structure and fully recover their functions. However, most tissues in the body have limited regenerative capabilities, and regeneration does not necessarily take place even though the tissues may have the potential to do so. Instead, wounded tissues are sometimes replaced by dysfunctional ones, such as scars [4], or by no tissue at all, leaving open chronic wounds [5]. Other pathologies additionally involve tissue morphogenesis, for instance, tumor formation in cancer [6].

The detailed characterization of developmental and regenerative morphogenetic processes provides a strong inspiration to the field of regenerative medicine and tissue engineering, which aims to replace or heal damaged or dysfunctional tissues for clinical applications. Currently, many tissue engineering strategies rely on the mimicry of physiological tissue morphogenesis, by delivering cells, material scaffolds, bioactive signaling molecules, a combination of those, or even more complex structured tissues, in place of defective tissues or organs [7]. In this chapter, we will give the reader an overview of key morphogenetic mechanisms that one can recapitulate when engineering tissues. Particularly, we seek to impart a good understanding of the dynamic interactions between cells and with their microenvironment within tissues, as well as between a tissue and the different physiological systems within the body, an understanding that is essential for tissue engineers.

Biology of tissue morphogenesis

As detailed in previous chapters, tissues are composed of cells and extracellular matrix (ECM). In tissues, cells constantly receive a multitude of signals, which can be from

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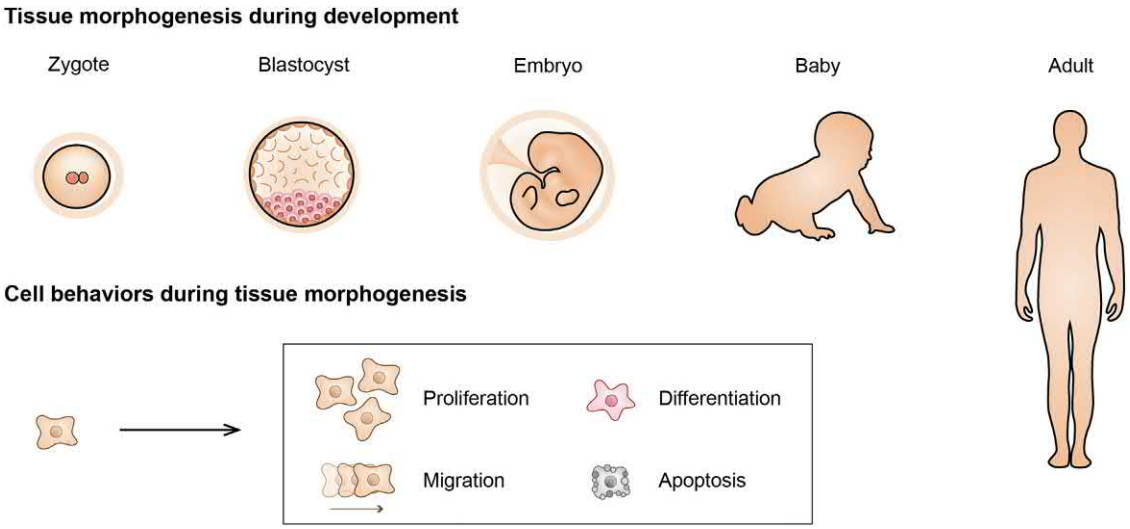

FIGURE 8.1 Overview of morphogenesis at tissue and cellular levels. Illustration of tissue morphogenesis occurring during the development of a unicellular zygote in a multicellular human adult organism. At the cellular level, tissue morphogenesis is the result of the coordination of multiple different cell behaviors, such as proliferation, migration, differentiation, and apoptosis.

soluble bioactive molecules, from the ECM via cell−ECM interactions, and from their neighboring cells via cell−cell interactions [8−10]. All these signals are integrated to instruct cell behaviors, which can simply remain at steady state or respond by a morphogenetic process (migration, proliferation, differentiation, apoptosis, etc.). In addition, when a cell modifies its behavior, its secretome and its interactions with the microenvironment and with neighboring cells are affected, which subsequently modify the signaling to other cells; this allows a tight spatiotemporal coordination of morphogenetic processes within the tissue [11]. In this section, we will first give the reader an overview of the molecular and cellular interactions involved during morphogenesis, before zooming out to a tissue-scale level, to highlight how tissues are interdependent and integrated in the body.

Morphogens as bioactive signaling molecules during morphogenesis

An important class of soluble molecules involved in tissue morphogenesis is called the morphogens [12]. They are bioactive signaling proteins secreted by cells that act in autocrine and paracrine fashion to control cell behaviors in the local microenvironment. Some of the morphogens are commonly referred as growth factors, as they are promitotic and thus induce cell proliferation or sprouting. For instance, the platelet-derived growth factor (PDGF), which is released in wounded microenvironment by platelet degranulation and secreted by immune cells, is a potent mitogen for mesenchymal cells, such as mesenchymal stem cells, fibroblasts, and vascular smooth muscle cells. PDGF is accordingly known to enhance tissue repair [12]. Moreover, growth factors oftentimes have multiple simultaneous biological activities and can, for example, trigger cell migration in addition to proliferation. In fact, PDGF also induces mesenchymal stem cell and fibroblast chemotaxis. Here, PDGF is taken as one example among many; various families of morphogens have been now classified (mainly based on their structural relation) and important efforts have been made to elucidate their biological roles during embryogenesis, tissue healing, and disease. As a consequence, many morphogens have been already characterized as being associated with particular mechanisms of tissue generation, including angiogenesis and lymphangiogenesis, neurogenesis and nerve sprouting, musculogenesis, osteogenesis and chondrogenesis, or in more organ-specific context, for example, hepatogenesis. Currently, in-depth studies on morphogens as well as their therapeutic use in regenerative medicine are under active investigation.

Morphogens are effective at a certain threshold concentration, commonly in the pico- to nanomolar range, which is considered a very low dose range [13]. More than the actual concentration, it has been shown that the formation of gradients of morphogens is fundamental to drive tissue growth in a coordinated way and induce cell patterning [13,14]. Gradient concentrations allow cells to receive direct spatial information about their position to a localized source (e.g., a secreting cell) (Fig. 8.2A). However, the question of how cells can perceive morphogens gradients and act accordingly is not yet fully elucidated and remains technically difficult to address. Morphogen gradient effects have been primarily studied in developmental biology; interestingly, it seems that the

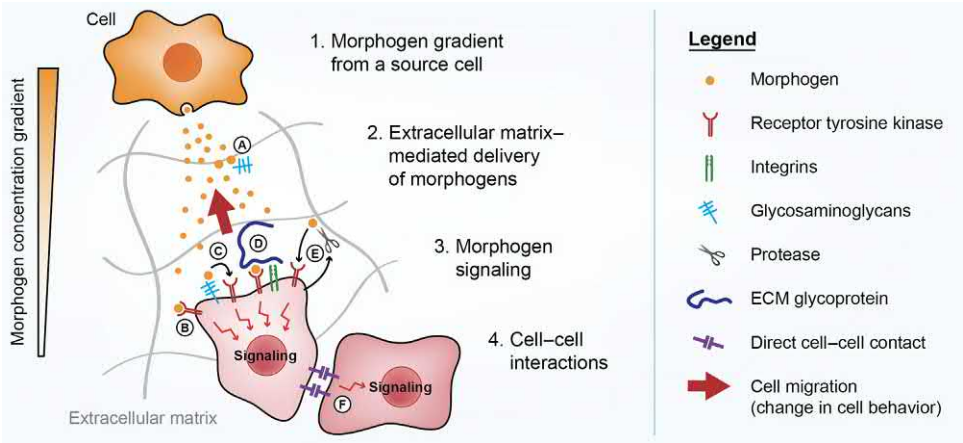

FIGURE 8.2 The ECM modulates morphogens release and signaling during tissue morphogenesis. Morphogens secreted by source cells create concentration gradients into the microenvironment, which are sensed by recipient cells. (A) Interactions between the ECM and morphogens control gradient steepness and morphogen release in the microenvironment. (B) At the cell surface, morphogens bind to their cognate RTKs and trigger intracellular signaling cascades to modulate gene expression and subsequent cell behavior. (C) Morphogens also interact with glycosaminoglycans (e.g., heparan sulfate) at the cell surface, from where they can be released to bind to their cognate receptors. (D) Clustering of RTK and integrins into complexes by morphogens bound to ECM glycoproteins can induce synergistic signaling that enhances morphogens regenerative effects. (E) Morphogens can additionally be released in a cell-mediated fashion, via the secretion of proteases that solubilize ECM-sequestered morphogens. (F) Morphogen-induced cell behavioral change can be communicated to neighboring cells via direct cell−cell communication, which allows behavioral coordination of multiple cells during morphogenesis. *ECM*, Extracellular matrix; *RTK*, receptor tyrosine kinase.

mechanisms of morphogen gradient sensing strongly depend on the particular morphogen of interest and even on the developmental stage. Among the main hypotheses, one stipulates that cells can have different response thresholds and so differentially respond according to their distance to the morphogen source. Furthermore, it has also been hypothesized that cells can sense concentration differences over their diameter, thus inducing cell orientation and polarization. While it remains unclear if cell fate determination is directly dependent on the presence of neighboring cells, it is thought that cells in the tissue can also regulate morphogen gradients through internalization and relaying such molecules and information to other cells, adding to the complexity of studying such mechanisms [14,15].

At molecular level, many morphogens activate cell surface receptor tyrosine kinases (RTKs) (Fig. 8.2B), which are strongly regulated by feedback loops to prevent a host of abnormal cell behaviors [16]. In the absence of stimulation by morphogens, RTKs accumulate at the cell surface, due to a faster endosomal recycling rate than their constitutive internalization rate. In contrast, upon activation, RTKs are internalized and sorted for degradation in the lysosomes, which reduces their presence at the cell surface and leads to cell desensitization [17]. Signaling through RTKs modifies cell gene expression, which can lead to alteration of the internal cellular structures, such as the cytoskeleton, and subsequent behavioral changes [18].

In addition to morphogens, the roles of other types of molecules are emerging in tissue morphogenesis; for instance, microRNAs have been shown to modulate multiple morphogenetic processes, notably in the context of brain development [19]. While not being the focus here, it is likely that the landscape of tissue morphogenesis signaling molecules will expand in the upcoming years.

The extracellular matrix as a key regulator of tissue morphogenesis

The ECM plays a major role during morphogenesis, in that it both regulates the spatiotemporal release of soluble morphogens to cells and provides cells with biomechanical and biomolecular signals through direct interactions [10,20,21]. First, morphogens can be specifically retained in the ECM depending on their affinity for ECM components. Many morphogens are known to bind to matrix glycosaminoglycans (GAGs) and are often referred as heparin-binding [22]. Because cell surfaces are also made of GAGs, morphogens can similarly be retained at the cell surface (Fig. 8.2C), before signaling to their cognate receptors. In addition to binding to GAGs, morphogens can also exhibit specific affinities for ECM glycoproteins, such as fibronectin, vitronectin, laminin, and fibrinogen [23−25]. Interestingly, fibrous collagens, which are core structural proteins of the ECM, do not seem to have strong interactions with morphogens. The specific affinities of morphogens for particular ECM components allow their temporal sequestration and release, as well as spatial patterning of the cell microenvironment. As such, the

ECM importantly regulates the formation and steepness of morphogen gradients in the microenvironment (Fig. 8.2A). Interestingly, it has also been shown that the biological effects of morphogens can be improved by the presentation by ECM glycoproteins. Indeed, ECM glycoproteins are multifunctional domain proteins that can display morphogen-binding domains close to cell integrin-binding domains. The proximity of these sites allows the copresentation of integrin ligands with morphogens, which can induce at the cell surface clusters of integrins with morphogen receptors. Such clusters can induce synergistic intracellular signaling, thus improving the biological cell response as compared to when morphogens are delivered without the copresentation (Fig. 8.2D) [26,27].

More than affinity-based release by the ECM, morphogens are also delivered by cell-mediated ECM degradation during morphogenetic processes. Upon cell migration or proliferation, cells secrete proteases that break down ECM components by enzymatic cleavage, which facilitates cell displacement in the tissue. By degrading the ECM, proteases importantly allow the release of matrix-sequestered morphogens (Fig. 8.2E) [28,29]. To counterbalance matrix proteases and protect the ECM from excessive degradation, cells in parallel secrete tissue protease inhibitors; the balance of both is responsible for regulating ECM degradation and renewal rates. In addition, matrix proteases not only accelerate the release of morphogens from the ECM, but they also unmask matrix biofunctional cryptic sites, which have been also shown to directly modulate cell behaviors by signaling through cell surface receptors. As an example, the cleavage of collagen IV by matrix metalloproteinase releases fragments that inhibit the proliferative effect of the vascular endothelial growth factor (VEGF) and migration of endothelial cells [30]. Exposure of matricryptic sites can also provide new adhesion sites for integrin receptors, to mediate direct ECM−cell interactions. For instance, at sites of inflammation, the thrombin-mediated cleavage of osteopontin renders available integrin $\alpha 9\beta 1$−binding sites that are involved in neutrophil migration [31].

In addition to degrading the ECM, cells are constantly renewing and remodeling it, which can lead to variation of the microenvironment composition and affect cell behaviors. For example, upon wounding, a provisional matrix made by fibrin clot primarily stops hemorrhage and is then replaced by a collagen III−based interstitial matrix, before being remodeled into a collagen I−based matrix over months [28,32]. The different compositions of the microenvironment define its biomechanical properties (e.g., stiffness, elasticity), which are transduced to cells by mechano-sensing cell surface receptors, such as integrins or cadherins, and directly affect cell responses; for instance, stiff environments can differentiate monocytes into myofibroblasts during scar formation [33,34].

Cell−cell interactions during tissue morphogenesis

Cell communication is essential to coordinate cell migration and structural organization during tissue morphogenesis. In tissues, cells usually connect to each other using stable cell junctions at steady state, namely, tight junctions, anchoring junctions, and gap junctions [8], although some cell types interact primarily with ECM components alone. Such junctions allow both exchange of biochemical signals between cells, through gap junctions, as well as biomechanical signals, mainly through cadherins in anchoring junctions. Importantly, cadherins have been shown to be critical for cell recognition and segregation during developmental stages, thus allowing tissue patterning; indeed, cadherins on one cell bind to the same cadherins on another cells (known as an homophilic binding), such that epithelial cells that exhibit E-cadherins can efficiently connect to other epithelial cells, but not to mesodermal cells, which display N-cadherins, for example [35].

Because cells thus communicate, the behavioral change of a cell in a tissue is necessarily perceived by its surrounding cells, which permits their coordination (Fig. 8.2F). As an illustration, during angiogenesis, the sensing of a VEGF-A gradient (secreted by hypoxic cells), by a specific endothelial cell, modifies its polarity and turns it into a tip cell, which migrates toward the source of VEGF-A. The tip cell induces a lateral inhibition in the surrounding endothelial cells, which then acquire a stalk cell phenotype. Stalk cells proliferate and follow the tip cell, leading to the formation of a blood vessel sprout that remains connected to the parent vessel [36]. As a second example, tissue growth is also highly controlled by mechanisms known as contact inhibition of proliferation and inhibition in locomotion [37]. Indeed, when cells collide, the formation of adhesion complexes at their membranes induces significant intracellular modifications, of their cytoskeleton notably, which inhibit their migration or proliferation and prevent pathological tissue growth. It is interesting to note that cancer cells are known to escape such regulatory mechanisms, which lead to the formation of tumors [37].

Tissues as integrated systems in the body

In the previous paragraphs, we detailed some key molecular and cellular interactions occurring during tissue morphogenesis. Here, we will rather replace tissue in the context of the whole body and discuss how tissues are interdependent upon one another. Such an aspect of tissue integration is particularly important to consider when engineering tissue, which will be the focus of the next section. Particularly, we will highlight the connections of tissues with the circulatory system, the immune system,

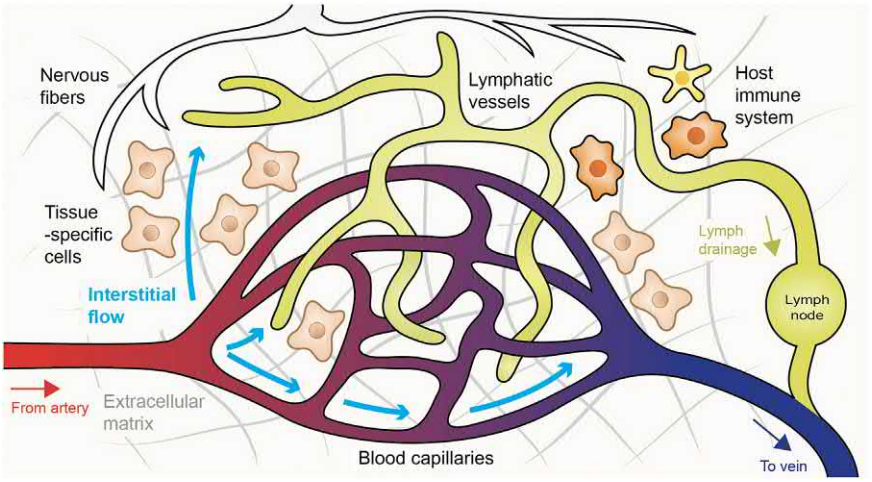

FIGURE 8.3 **Integration of multiple body systems in tissues.** In addition to tissue-specific structures, most of tissues in the body are irrigated by blood capillaries that deliver oxygen, nutrients, and signaling molecules to tissue-specific cells. Into the tissue the interstitial flow increases the bioavailability of such molecules to distant cells, which is further regulated by specific interactions within the ECM. Tissue fluid and metabolic cell waste are collected by veins and lymphatic vessels and return back to the systemic circulation. The immune and nervous systems, also present in most of the body tissues, fulfill important roles in maintaining tissue homeostasis, modulating or instructing tissue functions, notably via systemic feedback loop signaling, and in repairing tissue upon injury. *ECM*, Extracellular matrix.

and the nervous system, which are present in most of tissues (Fig. 8.3).

In addition to receiving signals from their local microenvironment, cells are affected by distant signals, primarily transported via the *circulatory system*. Almost all tissues in the body are irrigated by blood vessels, which bring oxygen and nutrients to cells and assist in regulating tissue temperature, to maintain cells alive. Furthermore, systemic signaling molecules such as hormones are secreted in the bloodstream to signal to distant cells. To reach cells within the tissue, molecules from the arterial blood either passively diffuse through the semipermeable vascular endothelial barrier or are actively transported from the apical to the basal side of endothelial cells by transcytosis [38]. Even outside of the vascular circulation, because the arterial circulatory system has a higher pressure than the venous one, tissues are constantly subjected to an interstitial flow [39]. This flow modulates the spatial and temporal patterning of the microenvironment, affecting molecular gradients and clearance, for instance [40]. Tissue fluid excess and cell metabolic waste are then mainly evacuated by the venous circulatory system. Another way through which tissue fluid and metabolic waste are drained is via the lymphatic system [41]. Like blood vessels, lymphatic vessels are also present in most of tissues and drain about 15% of tissue interstitial fluids (as compared to 85% for the venous system). The lymph collected by lymphatic vessels flows through lymph nodes before emptying back in the venous system at the cava vein level. In the case of pathogen invasion in a tissue, lymphatic vessels importantly allow antigenic molecule drainage and immune cell trafficking from the tissue to the lymph nodes, to efficiently activate the immune system [42].

Interestingly, the *immune system* is present and ensures surveillance in most tissues, which indeed contain circulatory and tissue-resident immune cell populations. The immune system plays a key role at steady state, in the context of healthy tissue renewal, in removing of apoptotic cells and debris by phagocytosis, as well as in immune surveillance, among other roles [43,44]. In context of inflammation, upon tissue injury, for example, immune cells secrete many cytokines and growth factors to recruit and instruct different cell types in the wound. In such cases, immune cells are essential to fight pathogens, clear tissue debris, resolve tissue inflammation, and guide subsequent tissue morphogenesis [45].

Finally, tissues are connected to the *nervous system*, with which they exchange sensory and motor signals to allow coordination at the whole-body level. Sensory fibers transmit efferent information from the tissue environment to the peripheral nervous system, and eventually the central nervous system in which signals from all tissues are integrated together. Information is relayed by many specific receptors, including proprioceptors, nociceptors, mechanoreceptors, thermoreceptors, and chemoreceptors, which allow precise monitoring of tissue changes. In response, motor fibers provide afferent motor signals to tissues, mainly to muscles and glands. In addition, the nervous system plays a role, not yet fully understood, in mediating tissue inflammation through modulation of the immune response, and so likely during subsequent morphogenesis [37]. It is also interesting to note that during development, the nervous and vascular circulatory

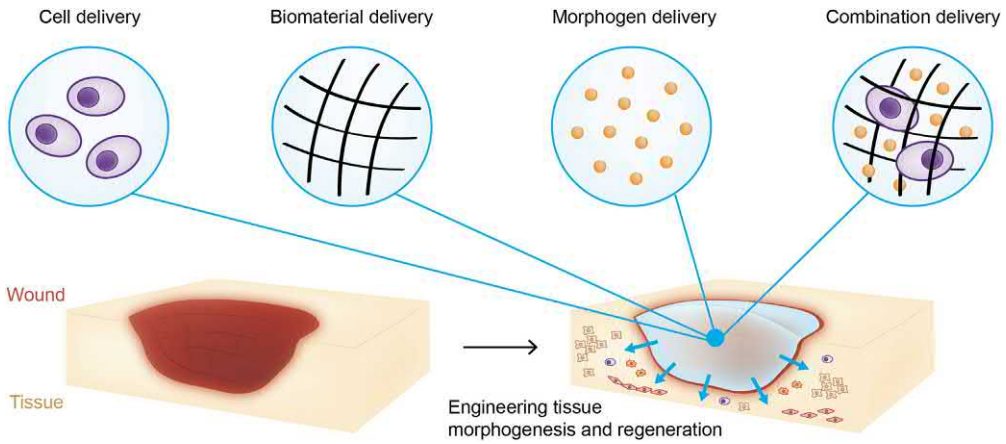

FIGURE 8.4 Common strategies to engineer tissue morphogenesis and regeneration. The field of tissue engineering develops many strategies to recover and repair tissue functions upon damage. To this purpose the delivery of tissue-specific or progenitors/stem cells, naturally derived or synthetic biomaterials, and bioactive signaling molecules as morphogens have been extensively studied, as stand-alone therapies or in combination. Such therapies aim at enhancing tissue regeneration upon administration, therefore inducing morphogenesis in the injured area.

networks share similar molecular signaling cues and codevelop together during tissue morphogenesis, each following the pattern of the other [46]. Consequently, the tight connection between the tissue and the circulatory, immune nervous systems controls tissue homeostasis and is essential to modulate the tissue response to environmental changes.

Engineering tissue morphogenesis

When engineering a tissue, one should keep in mind that it is the tissue functions rather than its structures that need to be ensured. However, because of the strong relation between tissue functions and structures, current tissue engineering trends are commonly based on biomimetic strategies that primarily aim at reproducing the physiological tissue morphology. For instance, three-dimensional organ bioprinting is mainly focusing on recapitulating organ structures in vitro that would eventually become functional upon implantation [47]. Other strategies, such as the engineering of implantable regenerative biomaterials, deliver signals that promote wound healing in vivo by recruiting the appropriate cell types to induce structure reconstruction. Letting the physiological tissue regenerate its own structure, through morphogenesis, could be an appealing approach to simultaneously induce tissue functions, although it might sometimes lead to only partial or no functional recovery depending on the delivered signals as well as on the etiology of the original tissue defect. Consequently, finding efficient ways to engineer functional biological tissues in vivo remains an important health challenge to date, mainly addressed by the fields of tissue engineering and regenerative medicine. Currently, most strategies to engineer tissue morphogenesis rely on the controlled delivery of cells, biomatrices, and bioactive signaling molecules, such as morphogens (Fig. 8.4), or a combination of those.

Cells as building units in tissue engineering

As cells are the primary constituents of biological tissues, direct delivery of cells to enhance tissue regeneration has been extensively explored [48,49]. Tissue-specific autologous cells can be extracted from the patient and expanded in vitro prior to implantation into the wound site, to promote healing. As clinical examples, autologous cultured keratinocytes or fibroblasts are used for repairing skin in burn patients [50], and autologous chrondrocytes into cartilage defect to repair hyaline cartilage [51]. Approaches based on the delivery of autologous cells have the strong advantage of being immune compatible to the patient and so would not be immune rejected upon delivery. Despite relatively good efficacy and safety, the process of culturing patient-derived cells in vitro can be long and expensive [52,53].

As an alternative strategy, stem/progenitor cells can also be extracted from the patient and delivery into wounds, wherein they can further proliferate and differentiate into functional tissue-specific cells to improve tissue healing. Particularly, autologous mesenchymal stem cells (MSCs) can be isolated from a patient with relatively easy procedures, such as from the patient adipose tissue [54]. Mesenchymal stem cells have been shown to promote reconstruction of various tissues, particularly cartilage, bone and muscle tissues [55]. Nevertheless, although MSCs can be extracted, isolated, and implanted into the patient over a 1-day period, in vitro expansion might still be required to obtain larger amount of cells.

For regeneration of tissues from other origins, for neural tissues, for instance, the use of less differentiated

embryonic stem cells might be appealing [56], although the embryonic source of such cells remains a strong limitation of this approach. In 2006 the key discovery that pluripotent embryonic-like stem cells could be induced from adult somatic cells opened a new perspective on the use of such stem cells for clinical applications [57]. In addition, the development of stem or fetal cell line banks with high regenerative potential can provide an alternative [53]; however, in this case, the cells' human leukocyte antigens need to be matched to the receiving patient to avoid immune rejection, as they are not directly extracted or derived from the patient's cells. Nevertheless, this approach, less personalized, might be more feasible at large scale for tissue engineering purposes considering current costs and technical challenges associated with personalized induced pluripotent stem cell (iPSCs)-based therapies.

While the most common strategy for cell delivery is by local implantation in the wound, the interesting properties of stem/progenitor cells to migrate into inflamed tissue upon systemic intravenous administration is now explored to target wounded sites and modify the local environment without the need of directly accessing the injured site, which might require surgery. Currently, such strategies are being tested in research and clinical trials [58,59].

Biomaterial scaffolds as artificial extracellular matrices

The relatively poor viability and engraftment of cells upon delivery into tissue has encouraged the use of biomaterial carriers for more efficient cell delivery. Indeed, many cell types are naturally adherent and die through a process called anoikis when they lose adherence. In such cases, biomaterials serve as artificial ECM to provide an environmental context to the cells, thus protecting them and guiding their fate within the tissue to regenerate [49].

In addition to being beneficial for use in combination with cells to deliver, the delivery of biomaterials may promote regeneration of the local cells in situ and tissue in the wounded site. Indeed, the major role of the ECM has inspired the study of a multitude of biomaterials for wound healing, which can be naturally derived or from synthetic origins [10]. The most complex material in terms of composition and closest to physiological environment is decellularized matrix [60] or cell-derived matrices [61]. Such matrices are derived from living tissue treated to remove the cellular content, yet preserving the structure and composition of the extracellular environment. Interestingly, decellularized matrices contain cell adhesion sites, mechanical and biomolecular signals to instruct and guide tissue morphogenesis upon implantation [62]. Decellularized matrices are often derived from allogeneic or xenogeneic origins, yet the removal of cellular content allows for controlled low immunogenicity. Currently, decellularized matrices are approved and used in many clinical applications, including for regeneration of bone, periodontal tissues, tendons, muscle, and other soft tissues [63].

Other naturally derived materials are derived from purified extracellular proteins, such as collagen- or fibrin-based materials [10]. Both collagen and fibrin are core structural proteins of the physiological ECM during homeostasis and in wounds, respectively. Thus similar to decellularized matrices, such environments provide a structural scaffold to support cell adhesion and migration, and subsequent tissue remodeling upon implantation. Collagen dressings are, for example, used for skin repair in burn patients, where they promote functional skin tissue regeneration rather than scarring [64,65]. Fibrin materials, on the other hand, have been widely used as tissue sealants for sustaining blood coagulation to prevent perioperative bleeding, in many regards mimicking the natural role of fibrin [66].

Biomaterials can also be derived from nonproteinaceous content of natural extracellular matrices, such as GAGs. Hyaluronic acid (HA)−based matrices are used for application in skin and hyaline cartilage repair in the clinic, for example, as well as in cosmetics [67]. Particularly, HA materials have a strong ability to attract and retain a large amount of water, keeping wounds and tissues highly hydrated, which is beneficial for healing. Although HA is a natural material, it has the advantage of being able to be produced biosynthetically, which avoids the need of animal sourcing. Other types of natural materials derived from nonmammalian organisms are also being explored in tissue engineering, such as chitosan- or silk-based materials [68,69].

In contrast to materials derived from living organisms, synthetic biomaterials offer many advantages in terms of ease of production, batch-to-batch reproducibility, and manufacturing at large scale, which are important to consider for commercialization of clinical products [21]. However, synthetic materials are often required to be engineered for cell adhesion and degradability, for which they are not naturally optimal. Interestingly, such properties of synthetic materials, along with their stiffness, elasticity, and shape, can often be tuned independently of each other, which offers the possibility of developing highly controlled microenvironments to precisely guide different cell behaviors. As such, synthetic materials can be highly tailored to their targeted biomedical applications. As an example, polyethylene glycol (PEG)−based scaffolds have been engineered with the arginine−glycine−aspartic acid (RGD) peptide, which is the most common minimal sequence allowing for cell adhesion [70]. In addition, incorporation of protease sensitive domains into these

materials, such as plasmin or matrix metalloproteinase cleavage sites, accelerate cell-mediated degradation and favors subsequent tissue remodeling [71]. Nevertheless, incorporation of multiple different domains into synthetic materials to enhance their biological properties increases in parallel their complexity, which might further complicate the regulatory path to clinical approval.

Morphogens as signaling cues in tissue engineering

Another motivation for the use of biomaterials in tissue engineering is to enhance drug delivery. Among the drugs, morphogens are potent candidates in regenerative medicine, as they can actively recruit cells from the own patient into the defect area and/or instruct them to guide tissue regeneration. However, the success of morphogen-based clinical therapy remains limited to date, due to cost-effective and safety issues [72,73]. Indeed, morphogens have been used at very high doses (in the order of milligrams, whereas physiological doses is ranging in pico- to nanograms) to accelerate wound healing, which in parallel induce adverse tissue remodeling, ectopic tissue formation, and tumorigenesis in distant sites from the treated area.

Retrospectively, the key physiological role of the ECM in regulating morphogen delivery, acting both for their local retention and allowing gradient formation, has been largely underestimated during these first trials therapies. This realization fostered the use and engineering of biomaterials for increased local retention of morphogens. Currently, the sequestration of morphogens into biomaterials has been achieved with relatively good success in preclinical research by various strategies, either based on biophysical and biochemical retention (e.g., porosity, viscosity, charge, and hydrophobicity) or on bioaffinity-based retention via material functionalization with moieties exhibiting specific affinities for morphogens [28]. Notably, many morphogens possess a natural affinity to GAGs and are as such referred as heparin-binding growth factors; functionalization of materials with heparin can be then exploit to increase the local sequestration of these morphogens [74]. On the other hand, proteins from the physiological ECM, such as fibrinogen, fibronectin, or laminin, also contain domains with specific affinities to morphogens, which can be incorporated into biomaterials to increase morphogen retention [75,76]. Because proteins contain more signaling information than GAGs, they can actively present morphogens to the cell surface and subsequently modulate signaling during morphogenesis. Of particular interest here is the synergistic signaling between integrins and growth factors, highlighted in The extracellular matrix as a key regulatory of tissue morphogenesis, which can be exploited to increase morphogen bioactive effects. As an illustration, the incorporation of a recombinant fibronectin fragment fusing a growth factor−binding domain to an integrin-binding domain into fibrin hydrogels enhanced the efficacy of VEGF-A, PDGF-BB, and the bone morphogenetic protein 2 in chronic skin wound healing and bone regeneration [26].

Not only can the biomaterial be designed for increased retention of morphogens, but morphogens can also be engineered for increased retention into biomaterials [24]. Morphogens can be covalently attached into materials, for example, which is the maximal degree of retention that can be achieved [77]. However, direct conjugation of morphogens into materials can impair their bioactivity and bioavailability to cells. Consequently, some strategies rather focus based on site-specific conjugation of morphogens, by modifying the morphogens sequences with substrate domain that can be covalently cross-linked into the material. For instance, transglutaminase-mediated enzymatic crosslinking of VEGF-A into fibrin or engineered PEG hydrogels has been developed by the fusion of a transglutaminase substrate sequence at the N-terminus of VEGF-A. Further incorporation of a matrix metalloproteinase (MMP) cleavage site between the crosslinking substrate sequence and VEGF-A allowed for the release of bioactive VEGF-A upon cell-mediated hydrogel degradation [78]. Using similar protein engineering strategies, growth factors can also be engineered with domains allowing high affinity but noncovalent retention into biomaterials. For example, morphogens that do not naturally bind to GAGs can be augmented with a heparin-binding domain [79]. Furthermore, morphogens have been recently fused to a domain displaying superaffinity for multiple ECM proteins, allowing strong retention into natural ECM-based biomaterials such as fibrin matrices, but also into the physiological ECM itself, which allow direct bind onto tissue upon delivery [25], thus limiting the need of exogenous carrier biomaterials.

Finally, it is likely that proper tissue morphogenesis and regeneration require the delivery of multiple and sequential morphogens, to recapitulate the natural wound healing cascade events. Codelivery and sequential delivery of growth factors have been explored in preclinical research [80−82] but might face regulatory hurdles to get approved in the clinic. Indeed, highly complex materials or with multiple bioactive compounds are subjected to longer and more expensive regulatory paths, which strongly limit their clinical translation.

Tissue remodeling in healthy and diseased environments

Delivery of cells, biomaterials, morphogens (or other bioactive signaling molecules), or a combination of those

constitutes to date the mainstream of research and development for guiding tissue morphogenesis in tissue engineering applications. Importantly, it should be kept in mind that anything delivered into the body may induce a host body reaction to the delivered moiety. Particularly, immune reactions can occur against foreign cells, biomaterials, or recombinant proteins [83,84]. Such reactions modify the microenvironments into which tissue morphogenesis is to occur and need to be considered at early stage in the design of specific therapy. While immune reaction can be dampened or limited by the use of poorly immunogenic materials, some recent research focuses on how to modulate immune cell responses at the wound location to induce a proregenerative microenvironment, in the perspective of developing immunomodulatory-based regenerative therapies [85].

Furthermore, it has been correlated that patients with a dysfunctional immune system, due to disease or pathology notably, are subjected to impaired physiological wound healing and tissue morphogenesis. In diabetic ulcers, for example, the wound remains stuck within a chronic inflammatory phase, characterized by a high content of proinflammatory cytokines and immune cells and increased level proteases [86]. In such dysregulated environments, delivery of cells, materials or drugs, and subsequent tissue remodeling will likely not lead to the same outcomes as in acute wounds. Therefore it is essential to consider the targeted pathophysiological environment to treat when developing therapies for specific applications of regenerative medicine.

Current focuses and future challenges

The regeneration of fully functional tissue upon wounding or organ failure is the aspiration of tissue engineering, which remains a challenge to date. Historically, early stages of regenerative medicine have explored tissue grafting to replace functional tissue, which still constitutes one of the main current clinical practices. Nevertheless, our better understanding of tissue morphogenesis encourages the development of biomedical strategies that deliver specific living cells, materials, or bioactive drugs to instruct the patient's own tissue toward regeneration at more cellular and molecular levels. In addition to promoting recruitment, proliferation and remodeling of tissue-specific or stem cells, particular efforts in the field of tissue engineering are dedicated to promoting tissue angiogenesis, as increased vascularization is known to enhance tissue healing. Indeed, reconnection of engineered tissues to the host circulatory system ensures long-term viability of the implanted or remodeled tissue. Recently, interest has also risen on controlling the host immune system response in tissue engineering therapies, considering both the difficulty of preventing immune reaction onset upon delivery of biological materials, as well as the key role of the immune system in orchestrating physiological tissue morphogenesis. Finally, fully functional engineered tissue would additionally require restoring connections with the host nervous system. To date, however, neurogenesis has been more extensively explored in the context of peripheral or central nervous system repair, as a first priority. It is likely that in the future, neurogenesis and the role of the nervous system during morphogenesis will be addressed in a broader context in tissue engineering.

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Chapter 9

Gene expression, cell determination, differentiation, and regeneration

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Introduction

Studies of skeletal muscle development were the first to provide the principles for understanding the genetic and molecular bases of determination and differentiation. Molecular signals from adjacent embryonic structures activate specific genetic pathways within target cells. Families of transcriptional regulators are expressed in response to these signals that initiate important developmental processes within skeletal muscle precursors as well as in other tissues and organs. Both activators and repressors are essential to control the time and location in which development occurs, and self-regulating, positive feedback loops ensure that, once begun, development can proceed normally. An understanding of the mechanistic basis of the embryonic commitment to a unique developmental pathway, and the subsequent realization of the adult phenotype, is essential for understanding embryonic development as well as stem-cell formation and behavior and how these pathways might be manipulated for tissue engineering and therapeutic goals.

This chapter focuses on determination and differentiation, classical embryological concepts that emerged from descriptive embryology, and how understanding these has led to approaches to commitment of cells to new fates. It has been through the study of muscle development that the genetic basis of these processes was first revealed, laying out a mechanistic basis for understanding determination and differentiation. Following success in studies of skeletal muscle (reviewed in Ref. [1]), genetic pathways involved in the determination of other cell types, some of which are detailed in other chapters in this book, have also been uncovered, largely because of the underlying conservation of structure among the various effector molecules and mechanisms.

What is becoming increasingly clear is that stem cells are laid down in embryonic development concurrent with the formation of specific tissue types in most organs. These stem cells are the basis of tissue renewal and repair. They often are quiescent after embryonic and fetal development and only are activated in response to tissue renewal, damage, or cells loss or death. Like embryonic development, optimal deployment of stem cells is dependent upon cell−cell interactions such that the cellular environment restricts or facilitates stem-cell formation and activation.

Determination and differentiation

Determination describes the process whereby a cell becomes committed to a unique developmental pathway, which, under conditions of normal development, appears to be a stable state. In many cases, cells become committed early in development yet remain highly proliferative, expanding exponentially for long periods of time before differentiation occurs. Until recently, determination could only be defined post hoc. Prior to the discovery of transcriptional regulators, there were few markers to indicate whether or not a cell was committed to a unique phenotype. Thus determination was operationally defined as the state that existed immediately prior to differentiation, that is, before expression of a cell-type-specific phenotype. The identification of transcription factors that control differential expression of large families of genes changed this concept.

Determination and differentiation are coupled during embryogenesis, where a small number of initially pluripotent cells expand in number and enter pathways through which they form the diverse cell types of the adult. The process of differentiation describes the acquisition of the phenotype of a cell, most often identified by the expression of specific proteins achieved as a result of

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differential gene expression. The differentiated state of a cell is easily determined by simple observation in most instances, because most differentiated cells display a unique phenotype as a result of the expression of specific structural proteins. Skeletal muscle cells are an extreme example of this, having a cytoplasmic matrix filled with highly ordered myosin, actin, and other contractile proteins within sarcomeres—the functional units of contraction—giving the fibers their cross-striated pattern. As development proceeds, there is a narrowing of the possible final cell phenotypes that individual cells can adopt, with the final cell fate (set of genes expressed) determined by factors both extrinsic and intrinsic to the cell (Fig. 9.1).

Determination and differentiation to a skeletal muscle fate begin in the somites of the early vertebrate embryo. Within the embryonic somites, two distinct anatomical regions contain muscle progenitors. Changes in gene expression responsible for directing cells to differentiate along particular developmental pathways result from the response to stimuli received from surrounding cells and the specific set of genes expressed in the cell itself at the time of interaction. For example, cells of recently formed somites have the potential to form either skeletal muscle or cartilage in response to adjacent tissues, and the fate adopted by a cell is a result of somite-cell location with respect to adjacent structures—the notochord, neural tube, and overlying ectoderm—that produce signaling molecules that determine phenotype [2–4]. Specified by signals from the neural tube and ectoderm, the dorsal portion of each somite forms an epithelial structure, the dermomyotome, which contains the precursor cells of all skeletal muscles that will form in the vertebrate body (with the exception of those found in the head). The medial portion of dermomyotome contains those precursors that form the axial or paraspinal musculature surrounding the vertebral column, whereas those cells of the ventral–lateral portion of the dermomyotome undergo a process of delamination and migrate into the forming appendages to produce the appendicular musculature of the limbs and body wall [3,4]. While the muscle fibers that form from the different regions of the somite are nearly indistinguishable, myogenesis in the axial and appendicular muscles is regulated by different effectors, demonstrating the complexity of determination and differentiation in the early embryo. These interactions not only result in the activation of muscle-specific structural and enzyme-encoding genes but also insure the continued expression of specific regulatory transcription factors which stably maintain the differentiated state.

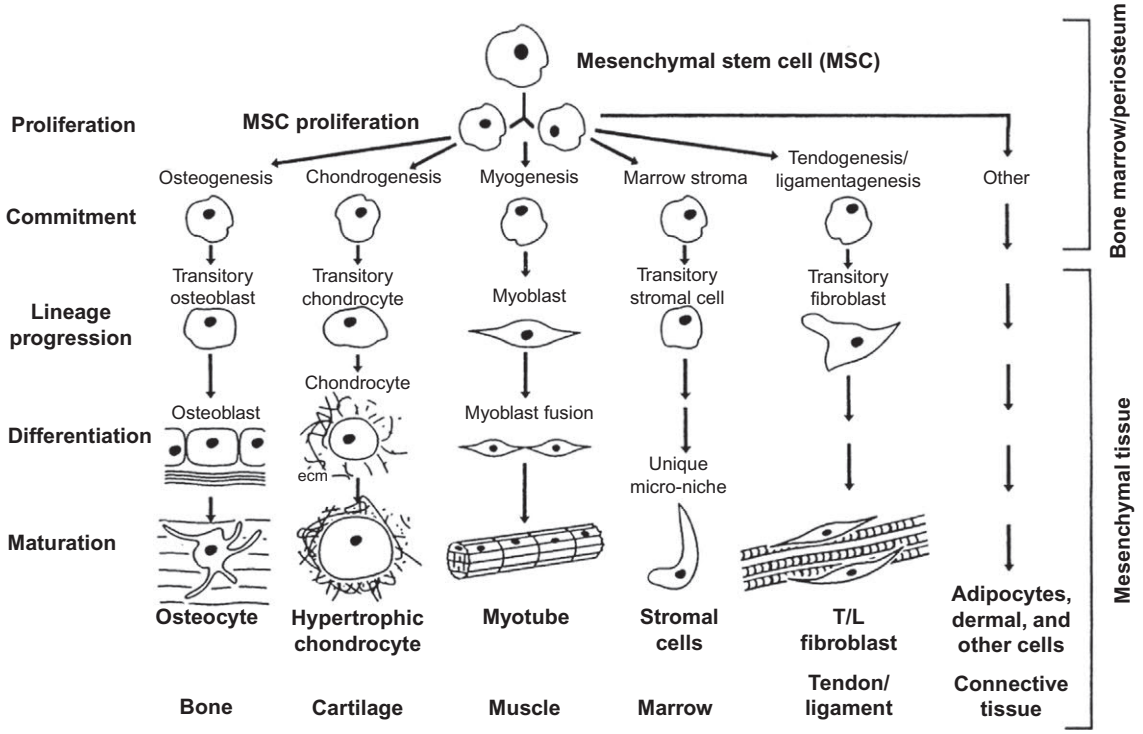

FIGURE 9.1 The process of commitment and differentiation.
Cells arise during gastrulation in the vertebrate embryo that subsequently produces all the different cell type of the body. Cells that can be designated as MSC proliferate and, in response to cues from the cellular environment, enter lineages that undergo differentiation and subsequent maturation into the mature cell types. *MSC*, Mesenchymal stem cells.

MyoD and the myogenic regulatory factors

It was not until late in the 20th century that investigators first demonstrated that cellular commitment to specific developmental fates could be determined by the expression of a single gene or a very small number of genes [5–9]. Improvements in tissue culture methods and rapid advances in molecular biology permitted the introduction of foreign genes into mammalian cells. The first gene capable of specifying a cell to a particular cellular phenotype was isolated and characterized in the laboratory of Weintraub [10,11]. They named this gene MyoD. Expression of MyoD is specific to skeletal muscle, and introduction of MyoD cDNA into fibroblasts of the 10T1/2 cell line converts them at a high frequency into stable myoblasts, which in turn differentiated into muscle fibers that express skeletal muscle proteins. MyoD was only the first of a family of myogenic regulatory factors (MRFs) to be discovered; others include Myf-5 [12], myogenin [13], and MRF4 [14]. Myf-5, MyoD, myogenin, and MRF4 are members of the basic helix-loop-helix (bHLH) family of transcription factors that bind to specific genomic sequences that control determination and differentiation of cells into skeletal muscle fibers during embryogenesis, postnatal myogenesis, and skeletal muscle regeneration [15]. This family acts in a hierarchical fashion that establishes the myogenic lineage as well as maintains the terminal myogenic phenotype during development and muscle regeneration [1].

Genetic experiments demonstrate that MyoD and Myf-5 establish the myogenic lineage, whereas Myf4 and myogenin mediate the expression of the terminal phenotype [16]. Muscle-specific transcription factors orchestrate the myogenic gene expression program by binding to particular sequences within the *cis*-regulatory elements of muscle-specific genes. [17]. These are short DNA motifs called E-boxes. The importance of MyoD and myf-5 to the determination of skeletal muscle was demonstrated when double knockout of these two genes in transgenic mice resulted in a nearly complete absence of skeletal muscle and myoblasts in offspring [16], whereas knockouts of one or the other do make skeletal muscle [18,19]. On the other hand, knockout of *myogenin* results in embryos with virtually no muscle fibers, but myoblasts do appear [20,21] suggesting that this gene is necessary for terminal differentiation. It is therefore believed that *MyoD* and *Myf-5* can compensate for each other and together act in a genetic pathway upstream of *myogenin* and *MRF4* to program all skeletal myogenesis. The role of the four MRFs has been reviewed [22].

MRF members share a common structure, a stretch of basic amino acids followed by a stretch of amino acids that form two amphipathic helices separated by an intervening loop (the bHLH motif). They are nuclear-located DNA-binding proteins that act as transcriptional regulators [1,17]. It has been demonstrated that the basic amino acids portion of each is required for DNA binding to the E-boxes and is essential for the myogenic conversion of fibroblasts to a skeletal muscle fate. The bHLH motif plays an essential role, because it is the basis for formation of heterodimers with other ubiquitously expressed bHLH proteins that facilitate DNA binding [17,23,24]. Sallee and Greenwald [25] reported that the ability of bHLH to activate lineage-specific programs of gene expression is so fundamental in differentiation that they were able to demonstrate bHLH proteins from humans activating the same functions in *Caenorhabditis elegans*.

As nature is conservative, it is not surprising that the bHLH motif was found in transcriptional factors, regulating the determination of cell types other than skeletal muscle. An example is the neurogenin family of bHLH genes. Based upon homology with MyoD, NeuroD (also known as BETA2 or NEUROD1 and 3) is one of the families of helix-loop-helix (HLH) transcription factors first isolated by the Weintraub laboratory and shown to act as a neuronal determination factor [26]. Introduction of the NeuroD gene into presumptive epidermal cells of Xenopus embryos or in human cells in conjunction with other transcription factors converts cells into fully differentiated neurons [27].

The neurogenins are a family of bHLH proteins expressed in neural precursors in the embryo prior to that of NeuroD, where they appear to function in facilitating the expression NeuroD. Determination of several different types of neuronal cells and the migration of neuronal cells are dependent on which neurogenin family member is expressed [28]. Neurogenins are known as the "proneural genes," because they are both necessary (by loss-of-function) and sufficient (by gain-of-function) to initiate the development of neuronal fate and to promote the differentiation of progenitor cells to acquire specific subtype identities [29]. It was subsequently shown that *neurogenin* 1, one of the three neurogenins, is required for the expression of a cascade of downstream bHLH factors, including NeuroD. Cells in different portions of the central and peripheral nervous are controlled by different members of this family and other bHLH genes. Ma et al. [30] have shown in the mouse embryos that loss of one of the three neurogenins, *ngn1*, leads to failure to generate sensory neurons of the inner ear. Like the MRFs, there are protein products of multiple bHLH genes, which activate other bHLH to control cell types. They act in both a positive and negative fashion and by interacting with other proteins to fine-tune differentiation [31,32].

Particular families of bHLH proteins can determine more than a single cell type. For example, NeuroD plays

an important role in the differentiation of pancreatic endocrine cells and of the retina in addition to other neural cell types [27,33–35]. In transgenic mice where NeuroD is knocked out, there are defects in multiple organs [36]. Pancreatic endocrine cells and neurons in the central and peripheral nervous systems die, resulting in cellular deficits in the pancreatic islets, cerebellum, hippocampus, and inner ear sensory ganglia. As a consequence, mice become diabetic and display neurological defects, including ataxia and deafness. These loss-of-function experiments suggest that the neurogenin family and NeuroD control expression of both common and distinct sets of molecules involved in cell survival and differentiation of different tissue types. But just like the MRFs, NeuroD does not function alone in neuronal differentiation and survival [37]. Additional bHLH proteins, including HES, Math-5, and Mash-1, have been shown to participate in the determination of neural cells as well. Differences in the expression of these various neurogenic bHLH proteins help to explain the diversity of neuronal cell types within vertebrate bodies. For example, genetic deletion of another bHLH protein encoded by the Mash-1 gene eliminates sympathetic and parasympathetic neurons and enteric neurons of the foregut [38].

Because cardiac muscle has so much in common with skeletal muscle, including a large number of contractile proteins, an exhaustive search was made for MyoD family members in the heart. Surprisingly, MyoD family members were not found in the developing heart, and thus they play no part in the differentiation of cardiac muscle cells despite the fact that many of the same genes for contractile proteins are expressed in cardiac as skeletal muscle cells. However, a different family of bHLH-containing factors, including dHAND and eHAND, were found in the developing heart. Unlike acting as determination factors, these bHLH factors are important for morphogenesis and the specification of cardiac chambers rather than for activation of contractile protein expression [39,40].

Negative regulators of development

In myogenesis and in other developmental pathways, there are both activators and repressors that act to modulate differentiation. These are found in both myogenesis and neurogenesis [41]. Acting as dominant negative regulators of the bHLH family of transcriptional regulators is a ubiquitously expressed family of proteins that contain the HLH structure, but lack the upstream run of basic amino acids essential for the specific DNA-binding characteristic of MyoD family members [42]. These transcription regulators are capable of dimerizing with MRFs. They are called inhibitors of differentiation (Id). These are a group of proteins that themselves do not bind to DNA but that can bind specifically with MyoD and bHLH products and in doing so attenuate ability of these proteins to bind DNA by forming nonfunctional heterodimeric complexes with them [42,43]. Thus in the case of myogenesis, they act to inhibit transcription of muscle-specific genes within proliferating myoblasts inhibiting the terminal differentiation program. Id levels decrease on terminal differentiation. Such associations with corepressor proteins and with proteins that titrate MyoD away from its DNA binding include not only the Id proteins but also other HLH proteins. These have been reviewed in Ref. [15,22].

Like myogenesis, neurogenesis is also regulated by repressors of the neurogenin family of bHLH activators [41]. An example is the HES family of HLH-containing proteins expressed in neural stem cells, where their presence prevents premature differentiation and permits proliferation of neuronal precursors. The interaction of unique sets of positively acting bHLH activators and negatively acting members of the HES family helps explain how different subsets of neurons undergo differentiation at different times during development, so that the complexity of the nervous system is produced [32,44,45].

MicroRNAs—regulators of differentiation

Activators of transcription and microRNAs (miRNAs) function during differentiation to modulate the expression of genes. Myocyte enhancer factor-2 (MEF2) proteins are a family of transcriptional activators that bind to A + T-rich DNA sequences found in several cells types and in many muscle-specific genes, including those encoding contractile proteins, muscle fiber enzymes, and the muscle regulatory factor, myogenin [46,47]. These are members of the MADs-box family of transcription factors. While some members of this family show a nearly ubiquitous distribution among tissue types, a few forms of the MEF2 family show expression that is more restricted to striated muscle. They do not act alone but act cooperatively with the MRFs in enhancing transcription of muscle-specific genes. Both MEF2s and MRFs bind to each other and to *cis*-modulating regions of the promotors of muscle-specific genes to enhance transcription [12,48,49]. So, while the MRFs are central to specification of cells to a myogenic fate, the MEF2 family acts synergistically to enhance transcription within the cells determined to this fate.

But not all regulation of differentiation is mediated by proteins. Another common method of control of differentiation is found in myogenesis and in most differentiating cells, which results in specification, maintenance, and regulation of complex regulatory circuits that have noncoding RNAs, including miRNAs [50]. Differentiation occurs not only through gene activation and transcription but also by suppression of transcription. The miRNAs sever

the suppressive function in differentiation at the posttranscriptional level. Rather than preventing or suppressing transcription, these RNAs prevent translation of messenger RNAs (mRNAs) within differentiating cells. miRNAs are incorporated into what are known as RNA-induced silencing complexes (RISC) that in turn alter the mRNAs that are translated [50]. A mature miRNA, incorporated into a RISC, guides the RISC to target complementary sequences usually present in the 3-UTR of mRNAs in the cytoplasm. The interaction of the miRNA, with its target site on the mRNA, typically has imperfect complementarity. The degree of complementarity influences the outcome of the interaction between the miRNA and its targeted mRNAs. The mechanism by which the miRNAs act is usually to repress protein translation by binding to specific mRNA, resulting in the mRNA ultimately being degraded.

Specific miRNA expression is initiated by the MRFs [51,52] to control production of skeletal muscle proteins within myogenic precursor cells. There are several miRNAs that are absent from undifferentiated myoblasts but are strongly upregulated upon differentiation [53]. MRF4 initiates the expression of miR-1 and miR-206. These miRNAs control translation of some of the muscle cell gene key transcripts. Experiments indicate that miRNAs can also modulate myogenic differentiation by affecting the translation of proteins not controlled by MEF2 or MRFs. An example is an increase of miR-1, a miRNA expressed in myogenesis. It accelerates myoblast differentiation by downregulating histone deacetylase 4 (HDAC4), a repressor of muscle differentiation [54]. By preventing the translation on HDAC4 mRNA, repression is released by miR-1 and differentiation proceeds. Thus by the repression of gene expression (transcription), for example, by Id proteins, the activation of gene expression by MRFs proteins, and the posttranscriptional repression of gene expression by miRNAs, the expression of characteristic of differentiating cells is fine tuned.

Pax in development

Much of what has been learned about the role of MyoD and myf-5 in the determination of skeletal muscle has come from studies on transgenic mice in which MRFs have been deleted [16,18–21,55]. The conclusion from this work was that MyoD and Myf-5 acted as redundant initial activators of myogenesis and as key players in commitment of cells in the early embryo to a myogenic fate. What then is the mechanism that initiates the expression of MyoD and Myf-5? Members of Pax family of transcription factor play this role early in somite maturation in the earliest stage of myogenesis. Pax3 and Pax7 are members of a DNA-binding family of proteins of primary importance to myogenesis. They bind to the regulatory regions of Myf-5 and MyoD and initiate their expression [56–59].

Pax3 plays the key role in early myogenesis while Pax7 predominates during muscle growth in the fetus and neonate and muscle regeneration in the adult [60]. In the double *Pax3*/*Pax7* mutant, cells fail to enter the myogenic program, leading to a major deficit in skeletal muscle [61]. Knockout of Pax3 alone leads to a complete absence of skeletal muscle, and the absence of MyoD expression and is the basis for placing Pax3 genetically upstream of the MRFs [62]. Thus while the MRFs control determination to the myogenic fate, members of the Pax family act downstream of these factors to dictate the expression of these master regulatory genes and thus to initiate entry into the myogenic program.

The second member of the Pax transcription factor family, Pax7, is a key element in the control of the growth of muscle and the potential for regeneration. Pax7 was isolated from satellite cells, a population of muscle-committed stem cells found in intimate association with mature muscle fibers. Satellite cells are responsible for muscle growth in the fetus and neonate and repair in the adult. Pax7 is specifically expressed in proliferative myogenic precursors, both embryonic myoblasts as well as satellite cells, and is downregulated at differentiation [63]. Transgenic mice lacking the Pax7 gene have normal musculature, with reduced muscle mass, but they have a complete absence or markedly reduced numbers of satellite cells [63,64]. These investigators found that in these Pax7 mutants, satellite cells, cells responsible for postnatal growth and regeneration of skeletal muscle, are progressively lost by cell death. These results suggest that specification of skeletal muscle satellite cells requires Pax7 expression or that Pax7 expression is responsible for survival of satellite cells. The interplay of the many factors that control the initiation and maintenance of myogenesis is shown in Fig. 9.2.

Satellite cells in skeletal muscle differentiation and repair

The reason that a discussion of skeletal muscle differentiation is so central to a basic understanding of cell differentiation is that this tissue unites concepts of tissue formation and tissue repair. The cell that unifies these is the stem cell of skeletal muscle called the satellite cell [65,66]. Among the first cells to be considered stem cells were the satellite cells of skeletal muscle initially described in 1961 by Alexander Mauro [67–69]. These cells lie just beneath the basal lamina that surrounds each muscle fiber within an anatomic muscle and above the plasma membrane of the fiber. These cells meet the definition of stem cells because they are self-renewing.

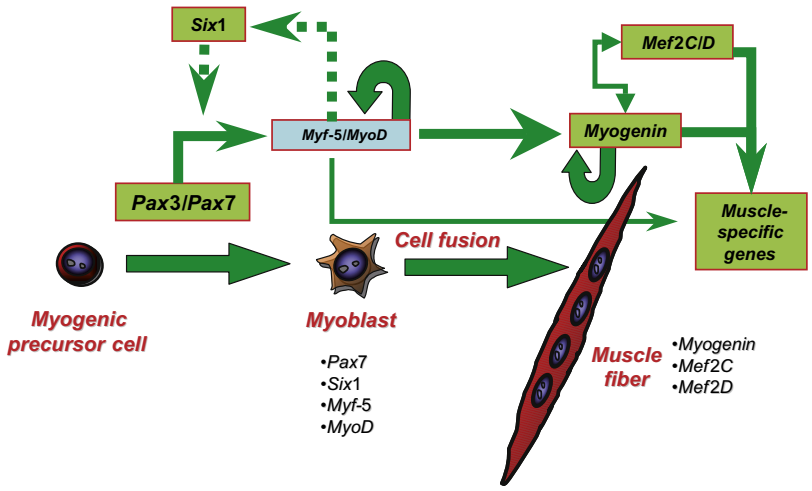

FIGURE 9.2 A regulatory network controls muscle cell differentiation. *Provided by Dr. Michael Rudnicki and modified.*

They are cells set aside in early muscle development, during which they contribute to fiber lengthening as fibers grow by cell fusion, as well as to the formation of additional fibers called secondary muscle fibers in the neonate [70] and to repair of muscle cells during regeneration. After neonate development, satellite cells remain quiescent unless fiber injury results in repair or regeneration. On the other hand, muscle hypertrophy, as in weight lifters, is primarily produced by expansion in size of existing fibers rather than fiber number. So satellite cells play little role in hypertrophy unless exercise injures fibers.

Satellite cells are located within special niches formed by the basal lamina. These niches keep satellite cells in a quiescent state until there is injury to the fibers of a muscle on which they lie. Injury results in the release of cytokines that initiate the proliferation and migration of the satellite cells on the surface of the injured fibers and on adjacent fibers. Satellite cells are distinguished from the nuclei of other cells within muscles because they express Pax7. The cell divisions of satellite cells are asymmetric—one daughter forms another satellite cell (stem cell) and is retained in the niche and the other goes forward as a myogenic cell that most often fuses with an injured fiber to reconstitute its multinuclear state, or occasionally, depending on the nature of the injury, to form a new fiber [71,72]. Thus while muscle regeneration is very limited in higher vertebrates, new fibers can form, and old fibers can be repaired while still retaining a supply of stem cells for future tissue repair. The challenge to investigators of tissue engineering with the goal of restoring muscle tissue is to develop strategies to facilitate extensive muscle stem-cell proliferation, cell survival, and efficient cell differentiation of transplanted cells. Attempts to expand normal myogenic cell populations for extended periods of time without losing ability differentiation in vivo have generally failed without transformation of the cells [73–75]. Equally challenging is gaining an understanding of the cells that form the connective tissue scaffolding of an anatomic muscle, if satellite cells are to successfully be used to treat neuromuscular disease, to combat ageing, or to reconstitute muscles following trauma [76,77].

Tissue engineering—repairing muscle and fostering regeneration by controlling determination and differentiation

Myogenesis is one of the most thoroughly studied systems where the detailed mechanisms of differentiations have been delineated. Skeletal muscle is also among the largest tissues in the human body. Because of its importance in ambulation, changes in muscle function place it in the forefront of clinical problems. Not only are there dystrophic diseases that compromise skeletal muscle function, but also this tissue is often severely damaged in civilian trauma and combat, and with ageing, muscle mass is lost. So, one would think that with such detailed understanding of how muscle cells originate, we would have strategies to combat these problems. Such strategies remain a challenge and only recently have studies revealed new options for employing our understanding of determination to solve problems of muscle bioengineering [77–79]. The principle challenges relate to the size of muscles when they need repair, the inability to produce large number of precursor cells for repair, the multiple cell types that must interact to produce an anatomically functional muscle, and the methods of delivery of cells for repair. Likewise, the three-dimensional aspects of muscle formation remain a challenge for tissue engineering [80].

Until recently, the most promising approaches to solve the problem of repair of skeletal muscle tissue damaged by disease or trauma were embryonic stem (ES) cells and

induced pluripotent stem (iPS) cells (iPSCs) [81]. There has been a flurry of experimental activity in model systems for regeneration and primary muscular diseases, which exploit these two types of cells [3,82–84]. ES cells are derived from the inner cell mass of preimplantation human and other vertebrate embryos. These cells can differentiate into a variety of cell types, including skeletal muscle, under the influence of factors added to culture media in which they are grown. On the other hand, iPSCs can be derived from many adult human cell types eliminating the ethical issues surrounding the use human embryos to derive stem cells. Using a limited number of genes, or by transfection with retroviruses, lentiviruses, or plasmids, such cells can be obtained through direct reprograming of human muscle cell types to propagate and to differentiate into muscle [82,85]. A downside of some iPS-like cells is that they can form teratomas when introduced in vivo [86]. Investigations in murine models have also identified a variety of other cells types that can differentiate into skeletal muscle, including bone marrow–derived side-population cells [87], mesenchymal stem cells [88], pericytes [89], $CD133^+$ progenitor cells [90], and mesoangioblasts [91]. These show a high myogenic propensity in vitro and in vivo, but their downside is that they are small populations of cells. In another approach, mouse and human satellite cells can be expanded under the influence of an inhibitor of a methyltransferase resulting in cell proliferation and can repopulate the satellite cell niche in normal and in preclinical models of muscular dystrophy [92].

Investigators have taken different approaches to produce cells that continue to proliferate, and ultimately differentiate, as well as give rise to cells with satellite properties [93]. In particular, these cells have been produced by manipulating the expression of PAX7 and MRFs, important factors in myogenic differentiation. But as noted in the discovery of the MRFs, the MyoD gene could directly convert fibroblasts cells into myogenic cells [10], but it was noted that these cells could not be propagated into large populations nor form satellite cells [94]. But investigators have shown that ectopic expression of MyoD, combined with exposure to small molecules, reprograms mouse fibroblasts into expandable myogenic progenitor cells [85]. These are designated as iMPCs. iMPCs express key skeletal muscle stem and progenitor cell markers, including Pax7 and Myf-5, and give rise to dystrophin-expressing myofibers upon transplantation in vivo into the muscle of mice with muscular dystrophy. iMPCs have the potential to (1) engraft in dystrophic and nondystrophic, injured muscles, (2) give rise to $Pax7^+$ cells (satellite cell like) within transplants, and (3) contribute to muscle regeneration in a serial-injury model. CRISPR/Cas9 technology could be used to restore dystrophin expression and such dystrophin-expressing iMPCs from patients with Duchenne muscular dystrophy could be used for cell therapy [85,95,96]. Thus human iMPCs may provide a source of cells for tissue engineering purposes to correct diseases or traumatic loss of muscle tissue.

There are advantages to using iMPCs or iPSCs in muscle repair. While the use of ES cells from humans is complicated by ethical considerations, iMPCs and iPSCs are not, because they do not require human embryos. These cells also have the advantage that each individual could have their own cells (autologous patient-specific stem cells) used to form iMPCs or iPSCs, thus obviating immunological rejection. But at least two hurdles remain to be overcome in tissue engineering of muscle repair—the size of muscles in humans anatomic muscles that require large numbers of cells for adequate repair and the array of other cell types that comprise a muscle. Ideally, transplanted myogenic cells must proliferate and persist in vivo and become co-opted into the satellite cell lineage for subsequent increase in fiber number or its repair. In any situation where cells for transplantation are encouraged to proliferate and differentiate in vitro following viral transfection, an important risk of problems rooted in insertional mutagenesis [97]. Despite these formidable problems, induced myogenic cells have become the favor cells for skeletal muscle transplantation. This approach has been exploited in several new models to form "muscles" where an anatomic muscle has had volumetric muscle loss [98]. Using gels and other matrices, human iPSCs can form three-dimensional "muscles" that perform contractile functions of normal skeletal muscle. Particularly interesting are studies that combine satellite cells, endothelial cells, hematopoietic cells, and fibro-adipogenic progenitors [99]. These can be combined with extracellular matrix proteins to form a hydrogel to attempt to restore function in models of volume loss. In these models of volumetric muscle loss, it has been shown that there is a return of many physiological functions to the damaged muscles, including innervation of the regenerating muscle. It has also been shown that the inclusion of an exercise program enhances structural repair, including innervation of the regenerating muscle [100–102]. Darabi et al. [97,103] and Mizuno et al. [104] have used induced cells from the mouse transfected with a gene (Pax7) fostering myogenic differentiation showing that these engineered cells are effective in engraftment, improving muscle function in disease models. As discussed by Rando and Ambrosio [77], engineering new muscles for repair attention has to be directed to the family of cell types and matrices that comprise an anatomic muscle. They make the point that efficient capacity to rebuild this tissue depends on additional cells in the local milieu, and without it, there is incomplete regeneration [79].

Conclusion

Determination and differentiation are in large part controlled by the expression of transcriptional regulators. These mechanisms begin early in development and involve the formation of cells that become committed to specific pathways of regulated gene expression. The regulators responsible were first characterized in studies committed to the differentiation of skeletal muscle development. The important proteins in the process are basic helix-loop-helix family proteins. Skeletal muscle development serves as a model for the mechanisms involved. Some other developing organs, such as the central and peripheral nervous systems, the pancreas, retina, and inner ear, employ very similar mechanisms and closely related members of the basic helix-loop-helix family of proteins to drive their development. But transcription alone does not control differentiation. Mechanisms that limit gene activation and transcription and miRNAs that restrain translations are crucial for the fine-tuning of differentiation. The progress in understanding differentiation of myogenic cells, their expansion in cell number, and transplantation techniques promises that basic understanding of determination and differentiation can lead to approaches to correct muscle fiber diseases and provide cells for muscle repair. Already our understanding of the mechanisms and effectors of determination and differentiation has been applied in strategies to engineer embryonic or adult-derived stem cells to address medical problems through transplantation. Particularly exciting are tissue engineering studies of transplanted myogenic cells combined with the many other cell types found in anatomic muscle and matrix gels as an approach to the problem of muscle volume loss.

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Part Two

In vitro control of tissue development

Chapter 10

Engineering functional tissues: in vitro culture parameters

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Introduction

Clinical disorders typically associated with musculoskeletal and cardiovascular tissues (i.e., osteoarthritis and myocardial infarction, respectively) often result in the loss of native tissue structural organization and mechanical function [1,2]. Tissue engineering is a rapidly growing field that seeks to restore the structure and function of tissues damaged due to injury, aging, or disease through the use of cells, biomaterials, and biologically active molecules (Fig. 10.1). Despite many early successes, there are few engineered tissue products available for clinical use, and significant challenges remain with regard to translating tissue-engineering technologies to the clinic for successful long-term repair of mechanically functional tissues. The precise reasons for graft failure are not fully understood, but they include a combination of factors that lead to the breakdown of repair tissues under conditions of physiologic loading.

Two critical aspects of tissue function in many tissue and organ systems are the transmission or generation of mechanical forces and the maintenance of blood circulation. In particular, articular cartilage (AC) and myocardium have highly specialized tissue composition and structure to allow for the specific mechanical and transport properties of these tissues. Although different in many respects, cartilage and cardiac tissues both perform critical biomechanical functions in vivo and lack intrinsic capacity for self-repair. Therefore cartilage and cardiac tissues are ideal targets for functional tissue engineering.

At the time of implantation, tissue-engineered constructs rarely possess mechanical properties that can

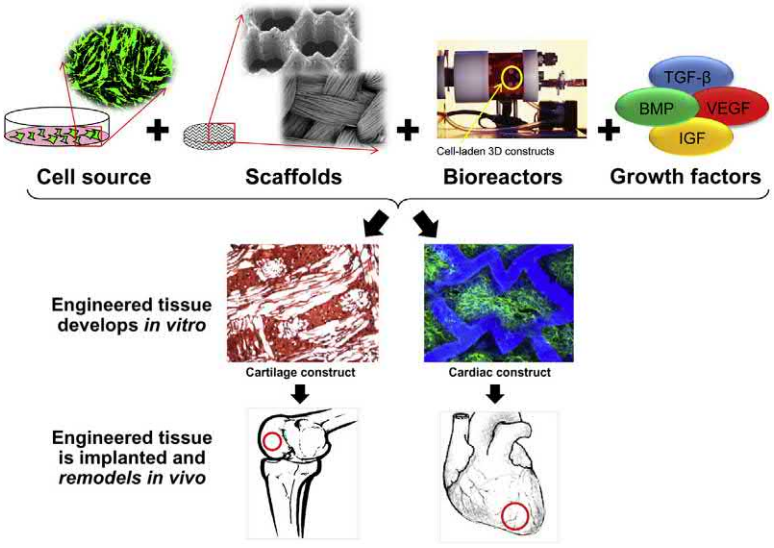

FIGURE 10.1 Strategy for engineering functional tissues in vitro.
Cells, scaffolds, bioreactors, and growth factors are used as tools to create functional engineered tissues. This chapter focuses on the in vitro culture of engineered cartilage and cardiac constructs that can be utilized for basic research and, potentially, for repair of damaged articular cartilage and myocardium. *Top panel: Scaffold images adapted from Engelmayr Jr GC, et al. Nat Mater 2008;7:1003 [3] (top) and Moutos FT, Guilak F. Tissue Eng, A 2010;16:1291 [4] (bottom). Middle panel: Engineered cartilage image adapted from Valonen PK, et al. Biomaterials, 2010;31(8):2193 [6]; engineered cardiac image adapted from Engelmayr Jr GC, et al. Nat Mater 2008;7:1003 [3].*

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withstand the high magnitudes of mechanical stresses experienced in vivo. For many native tissues, however, the potential range of in vivo stresses and strains are not well characterized, thus making it difficult to incorporate a true "safety factor" into the design criteria for engineered tissues. In addition, matching a single mechanical parameter, such as modulus or strength, is rarely sufficient, as most tissues possess complex viscoelastic, nonlinear, and anisotropic mechanical properties that may vary with age, site, and other factors [7].

A number of complex interactions must also be considered, as the graft and surrounding host tissues are expected to integrate and remodel in response to their changing environments postimplantation [8]. For example, convective transport of oxygen, nutrients, and waste products may become a limiting factor. Currently, most tissue-engineered constructs do not contain a functioning vasculature at the time of implantation, instead relying on either anastomosis of implanted capillary networks to the host vasculature or de novo angiogenesis to provide transport capability. Because oxygen has low solubility in aqueous media and diffuses over distances of only 100−200 μm, oxygen transport is often a limitation in cell survival within large, anatomically relevant grafts [9−11].

An evolving discipline referred to as "functional tissue engineering" has sought to address the aforementioned challenges by developing guidelines for rationally investigating the role of biological and mechanical factors in tissue engineering. A series of formal goals and principles for functional tissue engineering have been proposed in a generalized format [5,7,12]. In brief, these guidelines include the development of (1) improved definitions of functional success for tissue-engineering applications; (2) improved understanding of the in vivo mechanical requirements and intrinsic properties of native tissues; (3) improved understanding of the biophysical environment of cells within engineered constructs; (4) scaffold design criteria that aim to enhance cell survival, differentiation, and tissue mechanical function; (5) bioreactor design criteria that aim to enhance cell survival and the regeneration of functional tissue-engineered constructs; (6) construct design criteria that aim to meet the metabolic and mechanical demands of specific tissue-engineering applications; and (7) improved understanding of biological and mechanical responses of an engineered tissue construct following implantation. Since these guidelines were proposed, significant progress has been made toward developing in vitro culture systems and techniques to enhance graft mechanical properties and engineer functional tissues.

One of the key challenges in functional tissue engineering is optimizing the in vitro culture environment in order to produce three-dimensional (3D) implants that can meet the requirements of the in vivo milieu. In particular, the ability to precisely define and control in vitro culture conditions can be exploited to improve and ultimately control the structure, composition, and mechanical properties of engineered tissues. This chapter focuses on how advanced in vitro culture strategies, including the use of scaffold systems, bioreactors, growth factors, and mechanical conditioning, can influence the development and performance of engineered tissues (Fig. 10.1). Although the discussion addresses cartilage and cardiac tissues specifically, the concepts are also of relevance to other tissues and organs that serve some mechanical function (e.g., muscle, tendon, ligament, bone, blood vessels, heart valves, and bladder) and are the targets of tissue-engineering research efforts. The following sections of this chapter will consider key concepts, the importance of in vitro studies, and the influence of selected in vitro culture parameters on the development and performance of engineered tissues. Illustrative examples and alternative approaches for engineering cartilage and cardiac tissues are provided.

Key concepts for engineering functional tissues

Fundamental parameters for engineering functional tissues

Many tissue-engineering approaches involve the in vitro culture of cells on biomaterial scaffolds to generate functional engineered constructs. The working hypothesis is that in vitro culture conditions have a significant influence on the structural and mechanical properties of engineered tissues and therefore can be exploited to manipulate the growth and functionality of engineered tissues. In vitro *culture conditions* will refer to tissue-engineering scaffold systems, bioreactors, growth factors, and mechanical conditioning regimens that mediate cell behavior and functional tissue assembly [13,14]. *Scaffolds* will be defined as 3D material structures designed to perform some or all of the following functions: (1) promote cell-biomaterial interactions, cell adhesion, and extracellular matrix (ECM) deposition; (2) permit sufficient transport of gases, nutrients, and regulatory factors to allow cell survival, proliferation, and differentiation; (3) biodegrade at a controllable rate that approximates the rate of tissue regeneration under the culture conditions of interest; and (4) provoke a minimal degree of inflammation or toxicity in vivo [11,15]. *Bioreactors* will be defined as laboratory devices designed to perform some or all of the following functions: (1) provide control over the initial cell distribution on 3D scaffolds; (2) provide efficient mass transfer of gases, nutrients, and growth factors to tissue-engineered constructs during their in vitro cultivation; and (3) expose the developing constructs to convective mixing, perfusion, and/or mechanical, electrical, or other biophysical factors in a controlled manner [9,10,16−20]. *Mechanical*

conditioning will be defined as the in vitro application of dynamic mechanical loads (i.e., compression, tension, pressure, and/or shear) to cells, tissues, and/or 3D engineered tissue using custom designed systems. These fundamental in vitro culture parameters can be controlled independently or in combination to strategically meet the requirements of the specific tissue to be engineered.

Fundamental criteria for engineering functional tissues

The biological and mechanical requirements of an engineered tissue depend on the specific application. For example, engineered cartilage should provide a low-friction, articulating surface and be able to withstand and transmit load in compression, tension, and shear, whereas engineered cardiac tissue should propagate electrical signals, contract in a coordinated manner, and withstand dynamic changes in pressure, tension, and shear. In addition to tissue-specific requirements that serve as design principles for functional tissue engineering, there are fundamental criteria that all engineered tissues should meet (1) at the time of implantation, an engineered tissue should possess sufficient size and mechanical integrity to allow for handling and permit survival under physiological conditions; (2) immediately following implantation, an engineered tissue should provide some minimal levels of biomechanical function that should improve progressively until normal tissue function has been restored; and (3) after implantation, an engineered tissue should mature and integrate with surrounding host tissues.

Importance of in vitro studies for engineering functional tissues

Cells cultured in vitro tend to retain their differentiated phenotype and function under conditions that resemble their natural in vivo environment; these conditions may be generated using a combination of scaffolds, bioreactors, growth factors, and mechanical conditioning [5,7,12–14]. In vitro–grown tissue-engineered constructs can potentially be transplanted in vivo for tissue engineering and regenerative medicine applications or utilized as platforms for in vitro testing of cell- and tissue-level responses to molecular, mechanical, or genetic manipulations.

In vitro studies relevant to tissue engineering and regenerative medicine

In vivo models are essential for clinical translation of engineered tissues as they provide insight into the functional performance of engineered tissues. However, these models are complicated by high variability and biological and mechanical environments that differ from those existing in the human condition. To overcome these limitations, in vitro studies can be designed to understand the performance of the engineered tissue in vivo by (1) addressing the challenges of in vivo complexity in more controllable in vitro systems and (2) exploring how an in vitro–grown construct may behave when implanted in vivo. For example, tissue-engineered cartilage constructs pose a significant challenge with regard to variable and incomplete integration upon implantation in vivo [21,22]. To address the challenge of tissue integration, researchers have taken the approach of evaluating the integration of engineered tissues with native tissues using not only in vivo models [23] but also in vitro models (Fig. 10.2). In one study, cells were combined with three types of scaffolds [fibrin, agarose, and poly(glycolic acid) (PGA)], incorporated with explants of native AC and cultured as composites for 20 or 40 days [25]. The presence of native cartilage significantly altered cell proliferation and matrix accumulation in the composites. In addition, although engineered constructs based on all three scaffold materials adhered to the native cartilage, there were significant differences in the adhesive strength between the groups, suggesting that the type of scaffold may influence construct integration in vivo.

In another in vitro study, cells were combined with hyaluronan benzyl ester scaffolds, incorporated with three types of explants [native AC, vital bone, or devitalized bone (DB)] and cultured in rotating bioreactors for 4 or 8 weeks (Fig. 10.2) [24]. Engineered cartilage constructs interfaced with the solid matrix of adjacent cartilage without any gaps or intervening capsules. In addition, focal intermingling between construct collagen fibers and native cartilage collagen fibers provided evidence of structural integration (Fig. 10.2D–F). Interestingly, adhesive strength was higher for constructs cultured adjacent to bone than cartilage and highest for constructs cultured adjacent to DB (Fig. 10.2G). These findings could be explained by the differences in adjacent tissue architecture (histological features) and transport properties (diffusivity) [24]. Photochemical bonding has emerged as a potential tool for enhancing cartilage integration. Using the photosensitizer aluminum–phthalocyanine chloride, researchers were able to achieve improved shear strength between bonded native cartilage tissues, which persisted over a 1 week culture period [26]. Perfused bioreactors consistently yielded significantly higher amounts of glycosaminoglycans (GAGs) and total collagen in engineered cartilage cocultured adjacent to engineered bone than either engineered cartilage, native cartilage, or native bone [27]. These results suggest that the type of native tissue with which the engineered tissue is combined may influence construct integration in vivo. Collectively, information can be gleaned from in vitro studies with respect

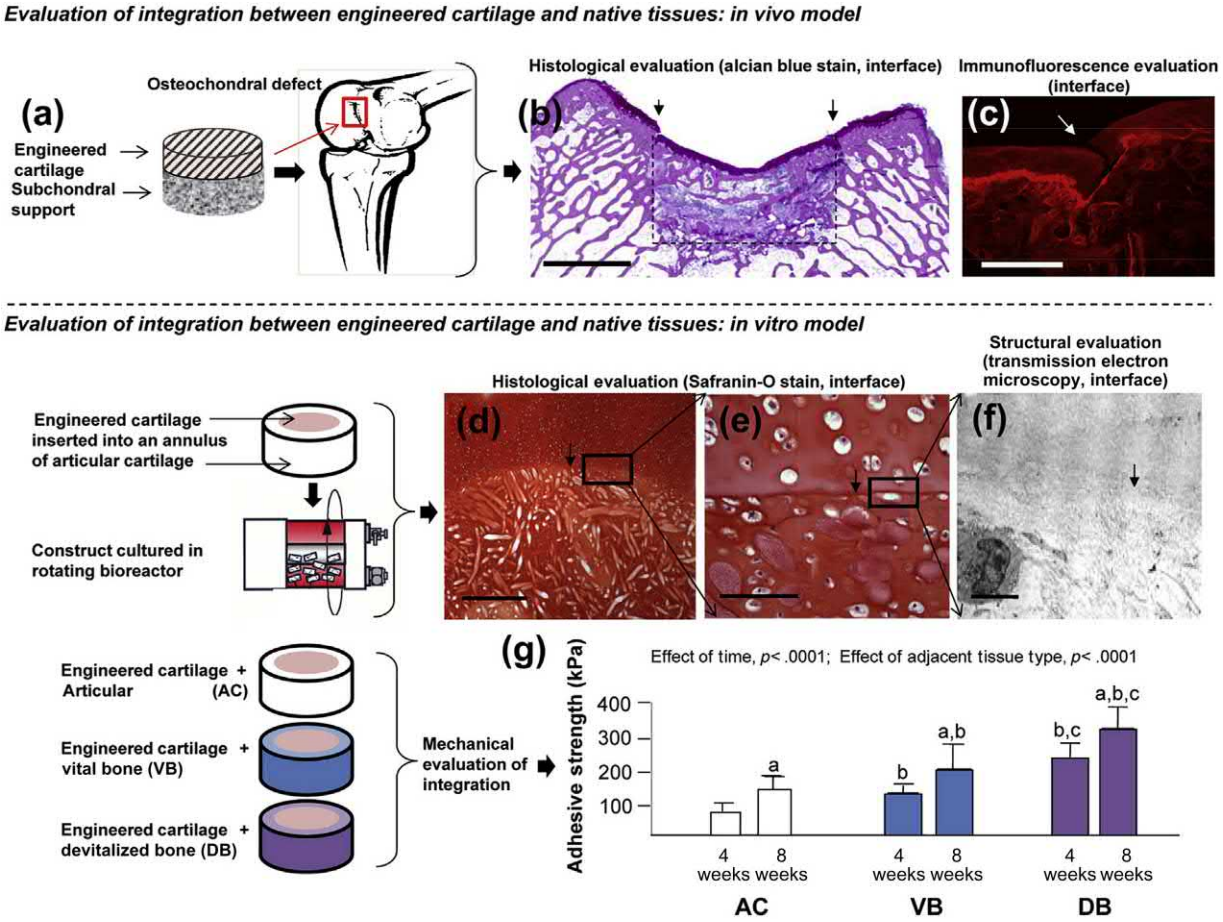

FIGURE 10.2 Studies of the clinically relevant problem of engineered cartilage integration.
Top panel: (A) Osteochondral implant integration was studied using adult rabbits with surgically created osteochondral defects. (B and C) Histological appearances of explants harvested after 6 months. (B) Alcian blue stain, scale bar: 2.5 mm, dashed line shows borders of original defect; (C) immunofluorescence stain, scale bar: 400 μm. Arrows indicate areas of incomplete integration between engineered and host cartilages.
Bottom panel: Engineered cartilage integration was studied using rotating bioreactors to culture engineered cartilage construct disks within rings of AC, VB, and DB. (D and E) Histology of the construct-AC interface (Safranin-O stain, scale bars: 500 and 50 μm for D and E, respectively). (F) Transmission electron micrograph of the construct-AC interface (scale bar: 5 μm). Arrows at interfaces point toward the construct and arrowheads indicate the scaffold. (G) Adhesive strength for construct disks cultured in rings of AC (white), VB (blue), or DB (purple) for 4 or 8 weeks. Data represented are mean ± SD. [a]Significant difference due to time; [b]Significantly different from the corresponding AC composite; [c]Significantly different from the corresponding VB composite. *AC*, Articular cartilage; *DB*, devitalized bone; *VB*, vital bone. *Top panel: adapted from Schaefer, et al.* Arthritis & Rheumatism*, 46(9) 2524, 2002 [23]. Middle and Bottom panels: adapted from Tognana, et al.* Osteoarthritis Cartilage *13(2):129, 2005 [24].*

to the integration potential of a tissue-engineered construct prior to in vivo implantation.

In vitro platforms relevant for high throughput screening of drugs and other agents

In vitro models can also be utilized to generate physiologically responsive tissue for screening pharmaceutical and therapeutic drugs (reviewed in Vandenburgh et al. [28]). This type of model can be designed for high throughput screening, thereby reducing the need for human tissue and organ harvest. Moreover, if based on human rather than animal cells (e.g., Schaaf et al. [29]), in vitro models can provide a more relevant system than in vivo animal models, which may differ significantly from the human case in their physiologic responses.

For example, a tissue-engineered drug screening platform referred to as "engineered heart tissue" was developed by combining neonatal rat heart cells with ECM components such as Matrigel and fibrin [30]. The cell−ECM−fibrin mixture was cast between two flexible posts such that maturation of the neonatal cells and condensation of the maturing tissue between the flexible posts yielded strips of cardiac-like tissue which contracted regularly. Pharmaceutical agents with known cardiac effects induced the expected changes within the

engineered heart tissue in a repeatable fashion, suggesting that engineered tissues of this type could be used for relatively high-throughput drug screening. Moreover, human embryonic stem cells (ESCs) were recently cultured to form engineered heart tissue that was successfully used to screen a panel of drugs with known proarrhythmic effects [29]. Engineered heart tissue may also be used as a surrogate cardiac tissue to explore in vitro the integration into native heart tissue of various cell populations, such as murine cardiomyocytes, cardiac fibroblasts, and murine ESC-derived cardiac cells [31,32]. These studies together suggest that engineered heart tissue can provide a valuable platform for screening cardiac pharmaceutical and cell therapeutics.

In vitro experiments, such as those described previously, are designed to elucidate cell- and tissue-level responses to molecular and mechanical stimuli and thus improve the understanding of complex in vivo phenomena and promote clinical translation of tissue-engineering technologies. In this context the in vitro culture environment plays a key role by allowing for controlled and reproducible test conditions that limit the variability associated with the in vivo environment.

Influence of selected in vitro culture parameters on the development and performance of engineered tissues

It is well-understood that traditional two-dimensional (2D) in vitro culture techniques cannot sufficiently recapitulate the microenvironment experienced by cells and tissues in vivo. Furthermore, physical forces are critical for the development of tissues and organs during embryogenesis and postnatal growth and remodeling. Therefore functional tissue-engineering strategies aim to recreate a 3D microenvironment in vitro that mimics the in vivo milieu through the use of scaffold systems, bioreactors, growth factors, and mechanical conditioning [5,7,12,14,17,33].

Culture duration

Cartilage tissue engineering

With the increase in vitro culture duration, chondrocytes assemble a mechanically functional ECM (e.g., Buschmann et al. [34]), and cardiomyocytes develop contractile responsiveness to electrical impulses (e.g., Radisic et al. [35]). For example, over a 40 day period of in vitro culture, constructs based on bovine calf chondrocytes and PGA scaffolds contained increasing amounts of GAG and type II collagen, and decreasing amounts of the PGA scaffold (Fig. 10.3A) [36]. In this system the relatively high rates of ECM synthesis and deposition by the calf chondrocytes approximately matched the relatively high degradation rate of the PGA scaffold, a finding that did not hold true when the same scaffold was studied with other cell types [e.g., bone marrow—derived mesenchymal stem cells (MSCs)] [14]. The structural and functional properties of engineered cartilage constructs can be improved to some degree by further extending the culture duration. For example, 7-month long cultures carried out in rotating bioreactors operated on Earth yielded engineered cartilage constructs with very high GAG fractions (~8% of wet weight) and compressive moduli (~0.9 MPa) that were comparable to normal AC (Fig. 10.3B and C), although the collagen fraction and dynamic stiffness of the 7-month constructs remained subnormal [37].

Optimal maturation of tissue-engineered cartilage in vitro may improve cartilage repair with respect to ECM quality and integration after implantation in vivo [38]. A recent study correlated engineered cartilage maturity with in vivo repair [39]. Engineered cartilage was prepared by

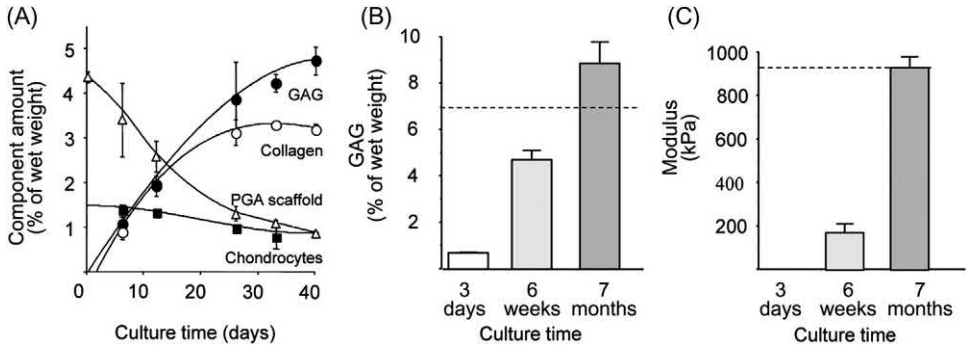

FIGURE 10.3 Effects of culture duration on the composition and function of engineered cartilage.
(A and B) Bovine calf chondrocytes were cultured on nonwoven PGA scaffolds in rotating bioreactors for up to 7 months. (A) Short term changes in the construct amounts of GAG (*closed circles*), total collagen (*open circles*), cells (*squares*), and PGA scaffold (*triangles*). (B and C) Long-term changes in construct amounts of GAG and Aggregate modulus (H_A), measured at 3 days, 6 weeks, and 7 months. Data are represented as mean ± SD. Dashed lines indicate average values obtained for native bovine calf cartilage. *GAG*, Glycosaminoglycan; *PGA*, poly(glycolic acid). *Adapted from (A) Freed LE, et al. Exp Cell Res 1998;58:240; (B and C) Freed LE, et al. Proc Natl Acad Sci USA 1997;94:13885.*

culturing chondrocytes on ECM scaffolds for 2 days, 2 weeks, or 4 weeks. Constructs were then implanted into full thickness cartilage defects in rabbit knee joints. In this study the use of more mature engineered cartilage improved osteochondral defect repair; however, another study showed that prolonged in vitro culture of engineered constructs prior to in vivo implantation may lead to ECM degradation, resulting in suboptimal performance in vivo [40]. Collectively, these findings suggest that while in vitro tissue maturation may enhance the in vivo performance of the engineered tissue, the optimal in vitro tissue maturation conditions are likely to be cell type-, scaffold-, culture condition-, and/or animal model-dependent.

Cardiac tissue engineering

Engineered cardiac tissue slowly forms contractile units over time in culture, beginning with spontaneous beating of single cells or groups of cells. As the neonatal rat heart cells mature, elongate, and form networks, coordinated, coherent contractions develop throughout the construct [41,42]. For example, engineered cardiac tissue cultured for up to 8 days exhibited a temporal increase in contractile amplitude [35]. In another study, engineered heart tissue exhibited coherent contraction at 7−9 days in culture, increased in force of contraction between 9 and 15 days in culture, and then stabilized [30]. Cells within these constructs showed an elongated phenotype consistent with more mature cardiomyocytes. Extended in vitro culture supports tissue maturation as shown in a study where IPSC-derived cardiac tissues gradually acquired electromechanical function over a 4-week culture period [43]. However, increasing culture duration alone is not sufficient to produce myocardium with the same functional properties as native tissue. At present, maximal contractile force generation reported for engineered cardiac constructs remains more than an order of magnitude below that of adult heart muscle [33].

Biomaterials

Cartilage tissue-engineering biomaterials

Novel 3D scaffold designs have attempted to mimic aspects of native ECM using composite scaffold structures. As biomimetic physical and mechanical properties are difficult to achieve with a single, homogeneous material, several approaches for tissue engineering have employed composite scaffold systems, which are often designed with fiber reinforcement and layered structures [11].

Fiber-reinforced constructs for cartilage repair

This type of design typically utilizes a fibrous phase comprises a nonwoven, knitted, or 3D woven fabric within a cell-supporting phase comprises a hydrogel or sponge-like material. The cell-supporting phase generally provides a favorable environment for proliferation, differentiation, and ECM synthesis, while the fiber phase functions as a mechanical reinforcement and stabilizer for the construct.

For example, hydrogels known to support chondrogenesis (e.g., agarose, alginate, and fibrin) have been combined with degradable nonwoven or woven 3D scaffolds to engineer functional cartilage. To test the influence of a hydrogel cell-carrier system for chondrogenesis, nonwoven poly(lactic-*co*-glycolic acid) (PLGA) meshes were seeded with either dissociated bovine chondrocytes or with chondrocytes suspended in alginate [44] and then implanted subcutaneously in nude mice. The alginate cell carrier increased seeding efficiency by assisting in the retention and uniform distribution of cells throughout the pores of the nonwoven mesh. The fiber-reinforced hydrogel also yielded a physically robust construct that maintained its initial geometry over time, without a negative effect on ECM synthesis. Similar studies showed success when nonwoven PGA was either combined with a chondrocyte-laden fibrin gel [45] or with MSCs in a type I collagen and alginate gel [46].

Woven 3D fabrics embedded with hydrogels have also been utilized replicate the complex biomechanical behavior of native AC [47]. A microscale 3D weaving technique was employed to fabricate multiple layers of continuous fibers in three orthogonal directions (Fig. 10.4A−D). Composite scaffolds comprise 3D woven fiber bundles of PGA [47] or poly(ε-caprolactone) (PCL) [4] were used in combination with fibrin gel to mimic the physical properties of native AC, specifically, its inhomogeneous, anisotropic, nonlinear, and viscoelastic mechanical properties. Construct compressive mechanical properties and equilibrium coefficient of friction were found to be similar to those of native AC throughout the defined culture period (Fig. 10.4E−G). Further, constructs seeded with human adipose-derived stem cells (ASCs) supported the elaboration of ECM which stained positive for the presence of chondroitin 4-sulfate and type II collagen (Fig. 10.4H and I). In other studies the infiltration of this 3D woven PCL scaffold with a slurry of cartilage-derived ECM enhanced the chondrogenesis of ASCs while providing a mechanically functional construct that resisted cell-mediated contraction [48]. A unique advantage of this composite structure is that scaffolds can be designed and fabricated with predetermined control of site-dependent variations in mechanical properties and porosity within a biocompatible matrix.

Fiber reinforcement can also improve the mechanical properties of sponge-like scaffolds that otherwise have insufficient mechanical properties to support mechanical loading. For example, a scaffold comprising a web-like collagen micro-sponge and knitted PLGA fabric was fabricated for engineering cartilage tissue [49]. The knitted fabric provided the mechanical integrity lacking in the collagen micro-sponge, and the collagen micro-sponge

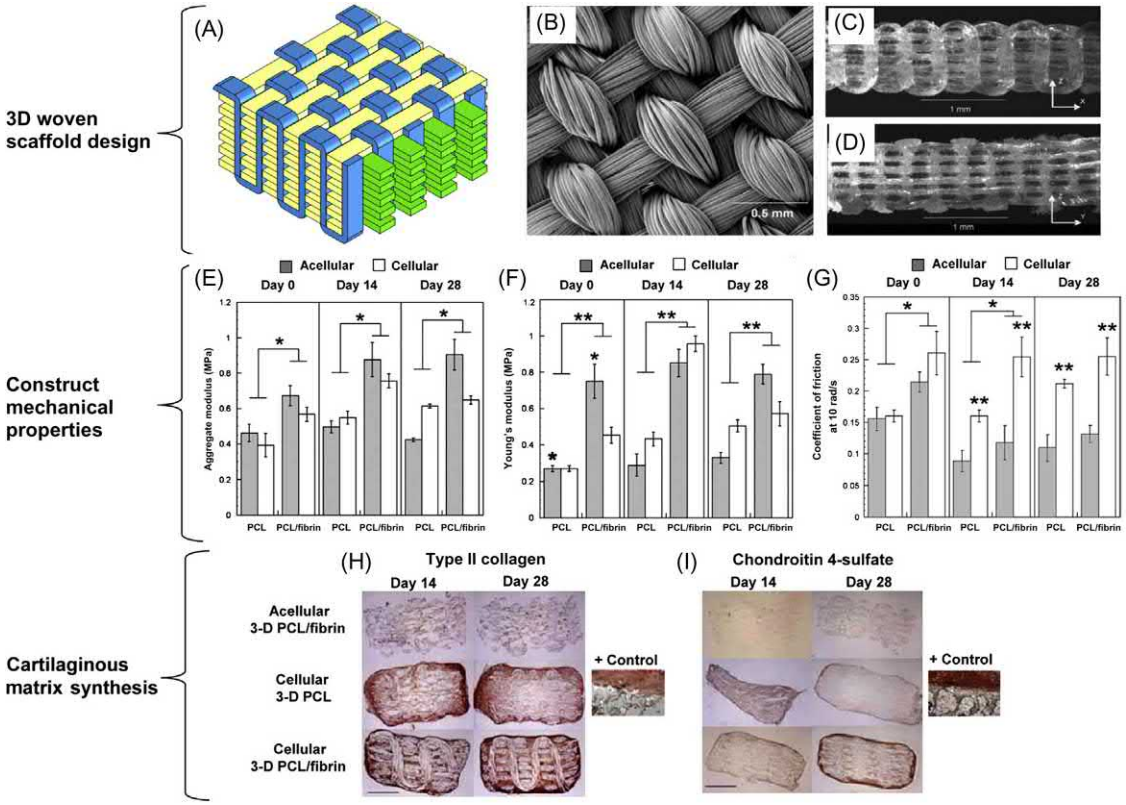

FIGURE 10.4 Structural and mechanical properties of a representative cartilage tissue-engineering scaffold.
Top panel: Fiber architecture of 3D orthogonally woven PCL scaffold. (A) Schematic and (B–D) scaffold weave and fiber morphology as shown with scanning electron microscopy [A—surface view, scale bar: 0.5 mm; B and C—cross-sectional view through the $X–Z$ plane (B), and $Y–Z$ plane (C), scale bar: 1 mm].
Middle panel: Compressive biomechanical properties of scaffolds without and with cultured cells at days 0, 14, and 28. (E) Aggregate modulus (H_A) and (F) Young's modulus as determined by confined and unconfined compression, respectively. The addition of fibrin to 3D PCL scaffolds significantly increased H_A and (E) for both acellular and cellular groups (ANOVA, $*P < .05$, $**P < .0001$). (G) Equilibrium coefficient of friction measured under steady frictional shear. Cellular constructs displayed significantly higher coefficients of friction against a rotating stainless steel platen than did acellular scaffolds (ANOVA, $*P < .05$, $**P < .001$). Data represented as mean ± SEM. Human adipose-derived stem cells used as cell source.
Bottom panel: Immunohistochemistry of acellular 3D PCL–fibrin composite scaffolds, cellular 3D PCL constructs, and cellular 3D PCL–fibrin composite constructs at days 14 and 28. (H) Type II collagen and (I) chondroitin 4-sulfate (scale bar: 1 mm). Human adipose-derived stem cells used as cell source. *3D*, Three-dimensional; *PCL*, poly(ε-caprolactone); *SEM*, scanning electron microscopy. *Adapted from Moutos FT, Guilak F. Tissue Eng, A 2010;16:1291 [4].*

filled in the large pores of the fabric to facilitate uniform cell distribution and cartilage-like tissue formation. Similarly, reinforced PLGA foam scaffolds were produced by embedding short PGA fibers into the bulk polymer prior to foaming [50]. The mechanical properties of these scaffolds could be tailored for potential use in AC repair by adjusting the material composition [51]. Collectively, this work demonstrates that fiber reinforcement is a controllable design variable that can be manipulated in order to engineer scaffolds to suit the load-bearing requirements of an engineered tissue.

Stratified and osteochondral constructs for cartilage repair

AC has a remarkably heterogeneous, intricate structure and composition. Indeed, Raman spectroscopic analysis reveals a far more complex zonal hierarchy than previously realized, identifying six noncalcified zones on the basis of biochemical content and organization [52]. In an attempt to recapitulate the structural, compositional, and mechanical complexity of AC, which varies with depth from the tissue surface, several groups have designed stratified constructs (reviewed in Klein et al. [53]). This has been approached by layering biomaterials or by creating biochemical/macrostructural gradients. In one study a two-layered construct comprising a top layer of 2% agarose and a bottom layer of 3% agarose exhibited zonal differences in the compressive elastic modulus immediately after fabrication [54–56]. Fabrication of layered PEG-based hydrogels comprises chondroitin sulfate, matrix metalloproteinase (MMP)-sensitive peptides, and/or hyaluronic acid (HA), promoted the differentiation of bone marrow–derived MSCs into chondrogenic

phenotypes that resembled those found in native tissue zones [57,58].

Zhu et al. were able to create biochemical and stiffness gradients in PEG hydrogels by chondroitin sulfate methacrylation [59]. Encapsulated chondrocytes and MSCs demonstrated graduated gene expression profiles in accordance with material properties.

The calcified zone in AC provides an important mechanical buffer to the underlying subchondral bone plate. Using a novel MSC and chondrocyte coculture system, Kunisch et al. reported the long-term maintenance of bilayered constructs comprising a nonmineralized and mineralized zone. The authors combined mineralized tissue disks formed by MSCs predifferentiated along an endochondral pathway with chondral layer comprising chondrocytes encapsulated within fibrin/PEG hydrogels [60].

Other groups have succeeded in engineering cartilage tissues with depth-dependent biochemical and biomechanical properties by layering chondrocytes isolated from specific tissue zones within PEG [55] and HA hydrogels [56].

Since bone-to-bone interfaces are known to integrate more effectively than cartilage-to-bone interfaces, osteochondral constructs are considered a promising technique for repairing full-thickness AC defects. Early osteochondral repair studies focused on engineering a cartilage layer which could then be combined with a bone graft substitute [23] or decellularized bone [61]. Issues concerning tissue integration and limited graft function prompted the development of multilayered, integrated constructs with or without cells. A diverse range of osteochondral constructs have been reported, typically comprising a polymer/hydrogel chondral layer and an integrated calcium phosphate containing osseous layer [62–67]. In trilayered strategy, Levingstone et al. [68] investigated osteochondral repair in a rabbit model using constructs composed of collagen 1-hydroxyapatite, collagen 1-HA, and collagen 1-collagen 2-HA. At 12 months CT and histological analyses revealed improved healing using multilayered acellular scaffolds compared to empty defect/single synthetic polymer scaffold controls [68].

Other approaches have aimed to spatially regulate osteochondral tissue formation within a single, homogeneous scaffold using chondrogenic and osteogenic growth factors [59] or by genetically modifying cells [69]. In the later study, simultaneous chondrogenic and osteogenic differentiation of MSCs cultured in singular culture conditions was achieved by transducing cells in the osseous layer with a dual lentiviral vector which resulted in the overexpression of the osteogenic transcriptional factor Runx2 and inhibition of SMAD3-mediated TGF-β signaling [69].

- In summary, advances in biomaterials design and fabrication techniques have supported the development of stratified chondral and osteochondral constructs enabling tissue formation to be regulated in a spatial manner. Combining osteochondral constructs with cells and/or biologics allows a greater level of control over cellular differentiation leading to improved biological and biomechanical performance.

Bioinductive and bioactive scaffolds

In addition to providing a suitable biophysical and mechanical environment, biomaterials may be functionalized to deliver biological molecules to cells. Such systems enable directed differentiation of implanted cells or promote the recruitment of endogenous cells following in vivo implantation. This may be achieved by immobilizing proteins directly onto materials [70,71] or encapsulating within microcarriers [72]. By layering materials containing specific growth factors, several groups have been able to spatially induce chondrogenesis and osteogenesis within a single construct. Lu et al. [72] combined gelatin microcarriers and oligo poly(ethylene)glycol fumarate hydrogels to investigate the effect of insulin-like growth factor-I (IGF I) and BMP2 delivery on osteochondral repair in a rabbit model. Another group used a minipig model to assess endogenous TGF-β3-driven cartilage repair using electrospun HA versus PCL scaffolds [70]. Inducible systems, for example, mechanically activated microcapsule delivery, offer the possibility to initiate growth factor/drug release within dynamic mechanical environments [73].

Biologics may alternatively be delivered using a gene-editing approach by immobilizing a viral vector encoding a desired transgene(s) onto a polymer or encapsulating within a hydrogel. Primary human MSCs are effectively transduced within fibrin hydrogels by adenovirus [74]. Delivery of adeno-associated vectors have been reported using a variety of biomaterials, including alginate–poloxamer composites [75] and poly-ethylene oxide and poly-propylene oxide copolymers [76]. TGF-β3-driven chondrogenesis of MSCs was achieved using a lentiviral vector immobilized onto woven PCL scaffolds [77].

Postimplantation, tissue-engineered constructs must tolerate dynamic and often unfavorable environmental conditions. As such, researchers may wish to build in control mechanisms enabling cells to respond to environmental challenges such as inflammation. In the case of synovial joints the cytokine interleukin-1 (IL-1) is a key orchestrator of inflammatory processes contributing to osteoarthritis [78]. By immobilizing lentiviral vectors encoding the IL-1 receptor antagonist (IL-1ra), one group have succeeded in generating inducible scaffold-mediated delivery of IL-1ra [79,80]. The use of scaffold-mediated vector delivery systems offers the possibility for spatial

control of cellular differentiation within a single-cell population by targeting multiple transgenes [81]. Such approaches have the potential to deliver functional cartilage repair together with drug delivery to diseased joints. By providing the appropriate biological cues, bioinductive/bioactive scaffolds enable more precise control over cell behavior. Inducible and tunable systems offer the possibility for patient-tailored implants, improving the functionality and clinical application of engineered tissues.

Cardiac tissue–engineering biomaterials

Scaffolds designed for cardiac tissue engineering must meet very different requirements than those developed for cartilage tissue engineering. Cardiac tissue–engineering scaffolds should provide the necessary microvascular and mechanical properties to meet the demands of a continuously contracting tissue, considering the cellular, geometric, mass transport, and oxygen supply concerns of native cardiac tissue. Scaffold approaches for cardiac tissue engineering have been reviewed by several groups [11,82–84] and are discussed briefly here.

In pioneering work, Ott et al. developed a biomimetic scaffold through decellularization of adult rat hearts [85]. Decellularized hearts were repopulated by intramural injection and coronary artery perfusion with neonatal cardiomyocytes and aortic endothelial cells. Under pulsatile flow and electrical stimulation, contractile tissue was generated in vitro. Although this system for cardiac tissue engineering has proven successful in many respects, the efficiency of recellularization remains low and the mechanical stiffness of the decellularized and recellularized tissues remains considerably higher than that of native myocardium.

Many natural and synthetic materials have been examined for use in cardiac tissue engineering. As described previously, Matrigel, combined with fibrin and/or thrombin [30,86–88] or with collagen sponges [35,89], has been used to generate contractile-engineered cardiac tissue in vitro. When considering the mechanical requirements of cardiac tissue, collagen gels and sponges may lack the mechanical strength required to withstand suturing and/or repeated cycles of stretch and relaxation. On the other hand, synthetic polymers popular in other tissue-engineering applications (e.g., PCL and PLGA) may be too stiff to form engineered cardiac tissue that possesses appropriate contractile properties. Therefore the development of other scaffolds that more closely mimic the native structural, mechanical, and transport properties of cardiac tissue is an area of active research.

In order to design a scaffold to address some of the specific structural and mechanical requirements of cardiac tissue, an elastomeric scaffold with accordion-like honeycomb pores was microfabricated from poly(glycerol sebacate) (PGS) (Fig. 10.5) [3]. The accordion-like honeycomb pores were chosen as a first step toward mimicking the native structure of collagen within the myocardial ECM (Fig. 10.5A–C). These scaffolds, when combined with neonatal rat heart cells, yielded constructs with anisotropic mechanical properties that closely matched to the right ventricular myocardium, coordinated contractions in response to electrical stimulation, and allowed for some degree of elongation and alignment of the neonatal heart cells (Fig. 10.5D–G). In addition, finite-element modeling of these and other porous, elastomeric constructs allowed for the interrogation of the mechanical properties of various scaffold features [90].

Many other scaffold designs have also used PGS as an elastomer for cardiac tissue engineering. One study used laser cutting to produce microvascular channels in porous scaffolds for improved nutrient transport and oxygen diffusion [91], and another used PGS sheets either unmodified or coated with gelatin and subjected to mechanical stretch [92]. In a perfusion system where cardiomyocytes were cultured on laminin-coated PGS a significant correlation was found between scaffold stiffness and both contractile force and compressive modulus of engineered tissues [93].

As we strive to reproduce the complex structure and vascular network of the heart, researchers are combining multiple biomaterials with advanced scaffold fabrication techniques. Using poly methyl methacrylate beads and polycarbonate fibers, Madden et al. [94] fabricated bimodal scaffolds containing interconnecting microchannels permissive for vascular invasion. Architecturally, the scaffolds resembled that of native myocardium, supported the proliferation of ESC-derived cardiomyocytes and were able to integrate into rat myocardium following in vivo transplantation [94]. In another study, researchers synthesized a novel polymer poly(limonene thioether) to generate multilevel porous scaffolds using micromolding and porogen templating. Cocultured vascular and heart cells were able to spatially organize on the scaffolds to form contractile, prevascularized tissue [95]. In terms of scalability the next step from engineered contractile tissues is cardiac chamber fabrication. To this end, scale models of the human ventricle were engineered by pull-spinning PCL/gelatin nanofibers into ellipsoidal chamber configuration and populating with rat ventricular myocytes/IPSC-derived cardiomyocytes [96]. Engineered ventricles demonstrated contractility and responsiveness to pharmacological stimulus; however the observation of poor calcium handling ability suggested immature myocardial development. These studies illustrate the importance of biomaterial mechanical properties, topographical cues, and scaffold architecture as critical determinants of functional outcome in engineered myocardium.

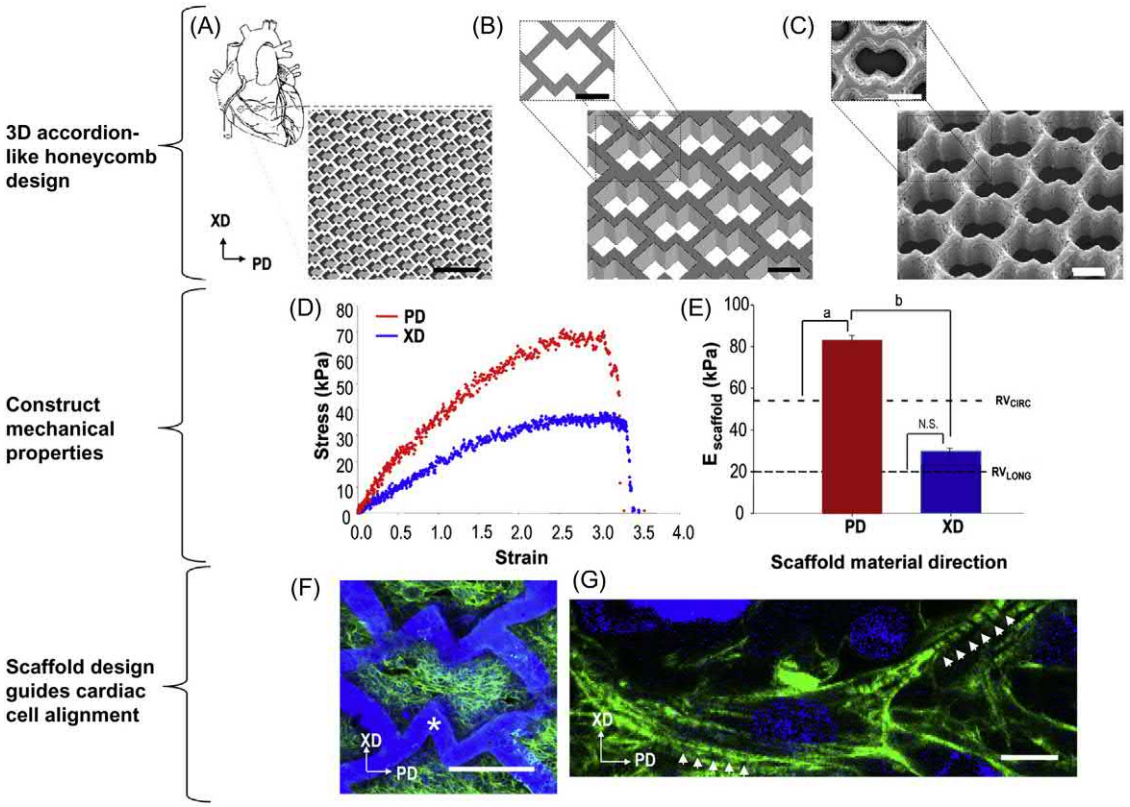

FIGURE 10.5 Scaffold design for engineering cardiac tissue.
Top panel: (A and B) Schematics of accordion-like honeycomb scaffold design (A—scale bar: 1 mm, B—scale bar: 200 μm). (C) Scanning electron micrograph of the scaffold architecture fabricated by laser microablation of a biodegradable elastomer, PGS (scale bar: 200 μm). PD and orthogonal XD material directions are indicated.
Middle panel: (D) Representative uniaxial stress−strain plot of PGS scaffold with cultured neonatal rat heart cells (scaffolds were fabricated from PGS membranes cured for 7.5 h at 160°C; cells were cultured for 1 week). (E) Scaffold anisotropic effective stiffnesses (E_{PD} and E_{XD}) are compared to specimens of native adult rat RV myocardium harvested in two orthogonal directions (RV_{CIRC} and RV_{LONG}, respectively). Data are represented as mean ± SD. [a]Significant difference between scaffold and RV; [b]Significant difference due to scaffold test direction; N.S. indicates not significant.
Bottom panel: (F and G) Confocal micrographs of neonatal rat heart cells cultured on accordion-like honeycomb scaffolds for 1 week. [Stain: filamentous F-actin (green), counterstain: DAPI (blue), scale bars: 200 and 10 μm, for F and G, respectively]. Scaffold indicated by white asterisks; cross-striations indicated by white arrows. *PD*, Preferred; *PGS*, poly(glycerol sebacate); *RV*, right ventricular; *XD*., cross-preferred. *Adapted from Engelmayr Jr GC, et al. Nat Mater 2008;7:1003 [3].*

Bioreactors and growth factors

Bioreactors, which are capable of initiating, maintaining, and directing cell growth and tissue development in a well-defined and tightly controlled culture environment, have proven to be crucial tools for 3D tissue culture. Tissue culture bioreactors represent a controllable model system for (1) studying the effects of biophysical stimuli and therapeutic agents on cells and developing tissues; (2) simulating responses of an in vitro−grown construct to in vivo implantation and thereby helping to define its potential for survival and functional integration; and (3) developing and testing physical therapy regimens for patients who have received engineered tissue implants. As described in the studies highlighted next, bioreactors have been used to (1) improve cell infiltration and distribution by dynamically seeding cells within 3D scaffolds, (2) facilitate cell expansion in order to mitigate unwanted phenotypic shift that may occur on tissue culture plastic, (3) overcome the limitations associated with oxygen and nutrient transport that are often observed in tissues cultured in static environments, and (4) enhance matrix synthesis and mechanical properties by biophysical stimulation of the developing constructs [16−18,20,97,98].

Cell seeding

Applying an appropriate cell type to a biomaterial scaffold is the first step in the tissue-engineering process, and the seeding technique may have a critical role in directing subsequent tissue formation [99,100]. Scaffold cell seeding has traditionally been done statically and performed manually using pipettes, but these static seeding techniques often lead to inefficient and spatially nonuniform

cell distribution within the scaffold [101], which may result in disparate ECM deposition throughout the construct [99,102]. In order to overcome the challenges associated with static seeding, bioreactor-based dynamic cell seeding techniques have been developed. Numerous types of bioreactors have been explored, including spinner flasks [103], wavy-walled reactors [104], and perfused vessels [105].

For some combinations of cells and scaffolds, dynamic cell seeding resulted in scaffolds with high and spatially uniform initial cell densities, which increased ECM deposition and compressive modulus in the resulting engineered cartilage [36,103,106]. For other combinations, perfusion of a cell suspension directly through a scaffold enabled spatially uniform seeding and enhanced tissue regeneration [101,105,107]. Engineered cartilage seeded in perfused bioreactors with alternating medium flow reportedly exhibited higher cell viability and uniformity than controls seeded statically and in spinner flasks [101]. Likewise, engineered cardiac tissue seeded in perfused bioreactors with alternating medium flow exhibited higher cell viability and spatial uniformity than controls seeded in mixed petri dishes [105].

With a view to scalable, clinical translation of engineered tissues, bioreactors are enabling researchers to streamline in vitro culture processing steps. By modifying perfusion rate, Tonnarelli et al. were able to perform cell seeding, expansion, and differentiation steps within a singular bidirectional bioreactor system for cartilage engineering [108].

Construct cultivation

Cellular apoptosis and the formation of necrotic regions within 3D engineered constructs cultured under static conditions suggest that diffusion alone does not provide sufficient mass transport of oxygen, nutrients, and wastes for cell survival within a construct. Bioreactors help to mitigate these mass transfer limitations and provide a controlled microenvironment for 3D construct development. Several groups have demonstrated that mass transport limitations could be minimized, cell viability, differentiation, and function enhanced, and matrix synthesis improved within engineered constructs with the use of bioreactors that induce convective mixing (spinner flasks [103,106] and rotating bioreactors [36,103,109]), and perfusion [17,18,89,105,110–113].

Convective mixing, flow, and mass transport are required to supply the oxygen, nutrients, and regulatory factors that are, in turn, required for the in vitro cultivation of large tissue constructs [9,10,114]. Different tissue types have different oxygen requirements, depending on cell type(s), concentrations, and metabolic activities. Oxygen is the factor that generally limits cell survival and tissue growth of engineered myocardium, due to its relatively low solubility, slow diffusion rate, and high consumption rate [10,115]. AC, on the other hand, an avascular tissue, has a lower requirement for oxygen than myocardium, a highly vascularized tissue. Experimental and modeling studies have correlated oxygen gradients within engineered tissues with morphology and composition [10,116,117].

Cartilage tissue-engineering bioreactors

A variety of bioreactors have been used to engineer cartilage constructs. Rotating bioreactors supported the growth of engineered cartilage constructs 5 to 8 mm thick based on bovine calf chondrocytes and PGS scaffolds [36,37,102,103]. More recently, human chondrocytes were expanded in 2D and then cultured on hyaluronan benzyl ester scaffolds in rotating bioreactors for up to 4 weeks [118]. While constructs cultured statically and in bioreactors contained similar amounts of GAG and collagen, the bioreactor-grown constructs exhibited a bizonal structure, consisting of a collagenous surface capsule deficient in GAG and an inner region that stained more positively for GAG. As compared to bovine calf chondrocytes, expanded human chondrocytes deposited relatively lower amounts of matrix. In another study a wavy-walled bioreactor was used to culture bovine calf chondrocytes on PGA scaffolds, and increased construct growth, defined by weight, cell proliferation, and ECM deposition, was observed in bioreactors as compared to spinner flasks [119]. Oxygen requirements for cartilage engineering within bioreactors are very much cell type dependent. While 20% oxygen tension is suitable for chondrocytes [120], lower oxygen tensions of $<5\%$ provide more favorable conditions for MSC chondrogenesis [121]. Hypoxia is a known regulator of MSC chondrogenesis in vitro [122] which is in accordance with oxygen gradient–driven lineage commitment of these cells during development [123].

In an effort to explore the influences of bioreactors and exogenous growth factors, an oscillatory perfused bioreactor providing slow, bidirectional perfusion was used to study cartilage constructs made by culturing adult human MSCs on 3D woven PCL scaffolds for 3 weeks (Fig. 10.6) [6]. Constructs cultured in bioreactors had higher aggregate moduli, higher total collagen contents, and similar GAG contents compared to constructs cultured statically (Fig. 10.6A and B). Constructs cultured statically in medium containing chondrogenic growth factors but not serum exhibited better chondrogenesis and more homogeneously positive matrix staining for GAG and collagen type II than otherwise identical medium containing serum (Fig. 10.6C–E). Constructs cultured in medium without chondrogenic growth factors and with

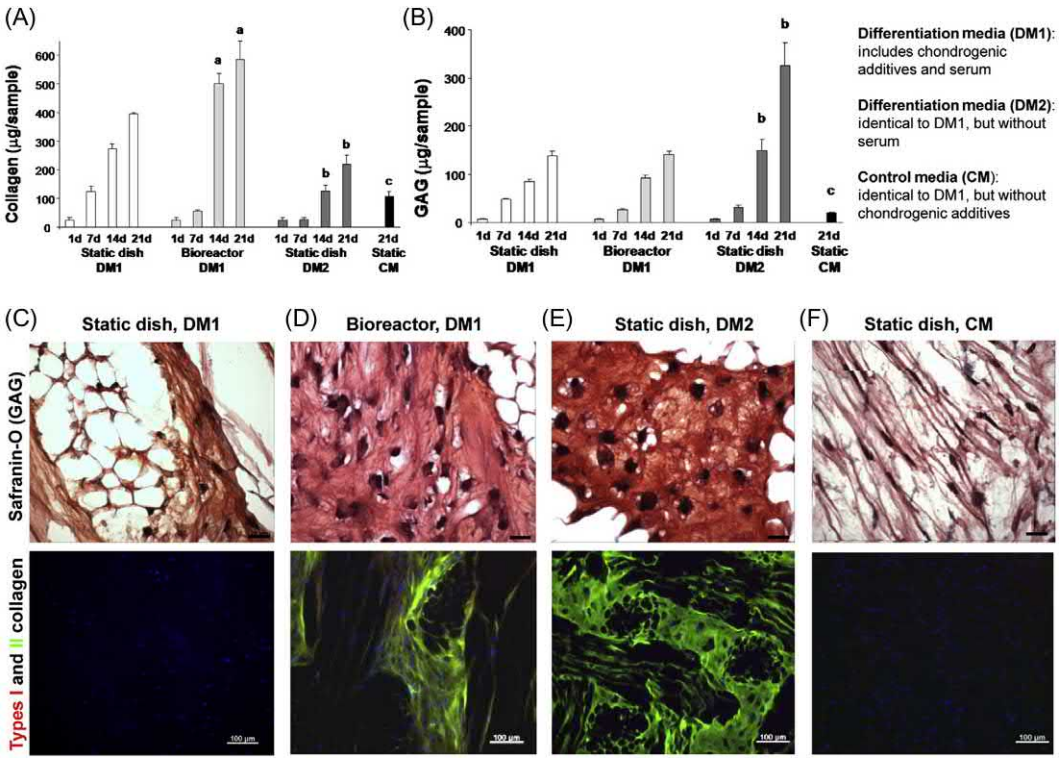

FIGURE 10.6 Bioreactors and growth factors influence the structure and composition of engineered cartilage.
Human MSCs were cultured on 3D woven PCL scaffolds in static dishes or bioreactors and in three different culture media (DM1, DM2, and CM) for up to 21 days. Top panel: Time evolutions of construct amounts of (A) total collagen and (B) GAG. Data are represented as mean ± SEM. [a]Significant difference due to type of culture vessel, [b]Significant difference due to presence of serum, [c]Significant difference due to chondrogenic additives, which included TGF-β3, (ITS), dexamethasone, and ascorbic acid.
Bottom panel: histological sections of 21-day constructs cultured (C) statically in DM1, (D) in bioreactors in DM1, (E) statically in DM2, and (F) statically in CM and stained with Safranin-O for GAG (top, scale bars: 20 μm) or immunostained for collagen type II (green) and type I (red, not seen) with DAPI (blue) counter-stain (bottom, scale bars:100 μm). *GAG*, Glycosaminoglycan; *ITS*, insulin-transferrin-selenium; *MSC*, mesenchymal stem cell; *PCL*, poly(ε-caprolactone); *SEM*, scanning electron microscopy. *Adapted from Valonen PK, et al.* Biomaterials*, 31(8): p. 2193, 2010 [6].*

serum did not exhibit chondrogenic differentiation (Fig. 10.6F). Together, these studies show that bioreactors and growth factors can influence cell morphology, proliferation, and ECM deposition in engineered cartilage.

Cardiac tissue-engineering bioreactors

In the case of engineered cardiac tissue, convective mixing in rotating bioreactors and spinner flasks supported the growth of a tissue-like surface layer ~100−200 μm thick [41,42,115]. Moreover, perfusion of culture medium directly through an engineered cardiac construct can significantly improve construct thickness and spatial homogeneity [89,105,110,115]. Specific design of perfusion bioreactors for cardiac tissue engineering is described in [17], and bioreactors designed specifically for electrical stimulation of cardiac constructs are described in [19]. In one example, perfused bioreactors enhanced the survival of heart cells cultured on porous collagen sponges by increasing the transport of oxygen and IGF-I (Fig. 10.7) [113]. Neonatal rat heart cells were seeded on scaffolds at high density by hydrogel entrapment, and then slow, bidirectional perfusion culture was carried out in an oscillatory perfused bioreactor for 8 days. Bioreactor grown constructs exhibited improvements over static controls with respect to several benchmarks, including reduced apoptosis, increased contractile amplitude, and increased expression of the contractile protein cardiac troponin-I (Fig. 10.7A−C). In the static control group (Fig. 10.7D), heart cells remained rounded and did not exhibit cross-striations, whereas in the bioreactor group (Fig. 10.7E), some cells were elongated and striated, albeit to a lesser degree than native adult rat ventricular myocardium (Fig. 10.7F). Moreover, perfusion of medium supplemented with IGF-I yielded further improvements in construct properties, presumably due to enhanced transport of growth factor to the heart cells within the 3D construct.

In another study, done in the oscillating perfused bioreactor, heart cells cultured on two-layered, 500 μm thick PGS scaffolds with fully interconnected accordion-like honeycomb pores exhibited increases in the gap-junctional protein connexin-43 and MMP-2, an enzyme associated

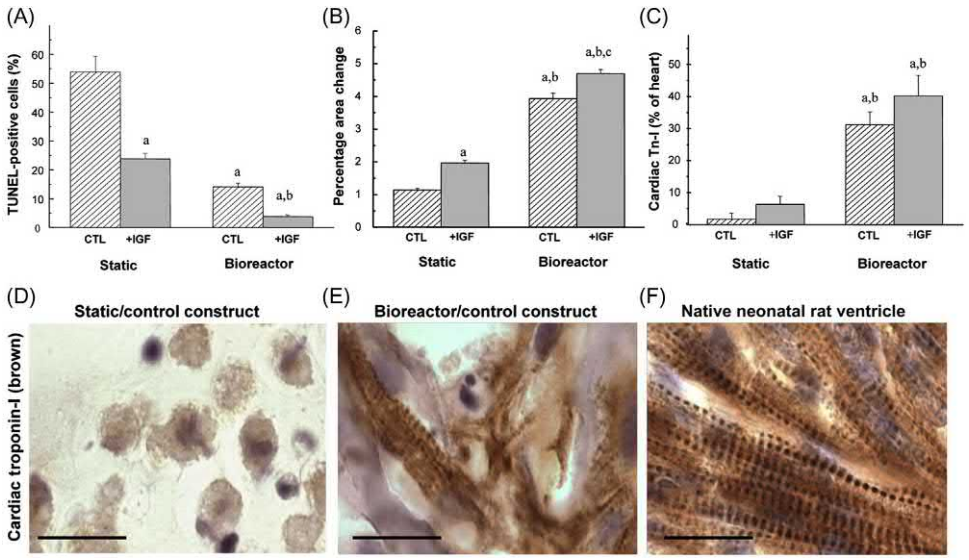

FIGURE 10.7 Bioreactors and growth factors influence the structure and composition of engineered cardiac tissue.
Neonatal rat heart cells were seeded in hydrogel and cultured on collagen sponge scaffolds either statically or in bioreactors in two different media (control, CTL, or with supplemental growth factor, IGF-I). Top panel: (A) Apoptosis (TUNEL-positive cells, percentage of total cells), (B) contractile amplitude (% area change), (C) cardiac troponin-I (Western blot, percentage of native neonatal rat heart). Data are represented as mean ± SEM. [a]Significantly different from static/CTL; [b]significantly different from static/IGF; [c]significantly different from bioreactor/CTL.
Bottom panel: Histological appearances of (D) static control construct, (E) bioreactor control construct, and (F) native neonatal rat ventricular myocardium immunostained for cardiac troponin-I (brown). Scale bars: 20 μm. *IGF*, Insulin-like growth factor; *SEM*, scanning electron microscopy. *Adapted from Cheng M, et al.* Tissue Eng, A*, 15(3): 645, 2009 [113].*

with tissue remodeling [124]. In other studies, sustained release and delivery of IGF [125] or sequential delivery of IGF-I and hepatocyte growth factor [126] enhanced survival and maturation of heart cells in a 3D model and protected against oxidative stress. Perfusion bioreactors are particularly useful for engineering and studying vascular structures. By combining cardiomyocyte-endothelial cell cocultured sheets onto vascular beds, Sekine et al. were able to generate fully perfusable vessels within a contractile tissue in vitro [127].

Together, these studies show that bioreactors and growth factors may work in tandem to enhance heart cell viability, contractility, and differentiation in 3D myocardial grafts.

Bioreactors and mechanical forces

Bioreactors can be used to apply biomechanical signals (shear, compression, tension, pressure, or a combination thereof) to growing tissues [128,129]. Chondrocytes are particularly responsive to mechanical signals and remodel the matrix according to the loads applied; thus the choice of loading regime can directly influence the development of the structure, composition, and mechanical properties of cartilaginous constructs (reviewed in Grad et al. [130,131]). Likewise, skeletal [132], smooth [133], and cardiac [33,86,87] muscle cells are quite responsive to mechanical signals.

Effects of hydrodynamic forces

Hydrodynamic forces associated with convective mixing can have a significant effect on the composition, structure, and properties of engineered tissues. For example, engineered cartilage cultured in rotating bioreactors had thinner surface capsules and higher fractional amounts of GAG than constructs grown in spinner flasks [103,109,134,135]. The flow field in the spinner flask was unsteady, turbulent (Reynolds number of 1758), and characterized by large spatial variations in the velocity field and maximum shear stresses [136]. In contrast the flow field in the rotating bioreactor (slow turning lateral vessel) was predominately laminar with shear stresses of ~ 1 dyn/cm^2 and a well-mixed interior due to secondary flow patterns induced by the freely settling constructs [134]. A model of tissue growth in the rotating bioreactor that accounted for the intensity of convection over 6-weeks of in vitro culture was used to predict the morphological evolution of an engineered cartilage construct [137]. In particular, the model predicted that high shear and mass transfer at the lower corners of a settling, discoid construct would preferentially induce tissue growth in these regions, and that temporal changes in construct size and shape would further enhance local variations in the flow field in a manner that accentuated localized tissue growth. The computed velocity fields and shear stress data corresponded well with the morphological evolution

of engineered cartilage, as shown by superimposing a calculated flow field on a histological cross section of an actual construct. The previous examples suggest that combining experimental studies and computational modeling of hydrodynamic shear stresses and concentration gradients in bioreactors may help to explain underlying mechanisms that control the growth of engineered tissue constructs.

Effects of mechanical tension, compression, and shear loading

It is well known that mechanical forces are critical for determining the architecture of native tissues, such as bone [138], and there is growing evidence that mechanical factors are important factors in determining stem cell fate [139]. In particular the role of in vitro mechanical stress in maintaining and promoting the chondrogenic phenotype has been the topic of several investigations, but the specific influences of different physical stimuli and their interactions with the biochemical environment are not fully understood. For example, dynamic compression caused a ~2-fold increase in cartilage nodule density and a 2.5-fold increase in GAG synthesis in stage 23/24 chick limb bud cells cultured in agarose gel [140]. Likewise, cyclic hydrostatic pressure significantly increased the amounts of proteoglycan and collagen in aggregates of human bone marrow–derived MSCs [141]. In another study, compression enhanced chondrogenic differentiation in mouse embryonic E10 stage cells embedded in collagen type I compared to unloaded controls, as shown by upregulation of SOX-9 and downregulation of IL-1β expression [142].

A variety of devices have been custom-designed and built to study the effects of mechanical conditioning (i.e., compression, tension, pressure, or shear) on cells and tissues in vitro (reviewed in Brown et al. [143], Darling et al. [144], and Waldman et al. [145]). For engineering cartilage, devices may apply dynamic compression (e.g., Buschman et al. [34], Mauck et al. [146]), hydrostatic pressure (e.g., Mizuno et al. [147], Toyoda et al. [148]), mechanical shear (e.g., Waldman et al. [149]), or a combination of dynamic compression and shear [150]. For engineering skeletal, smooth, and cardiac muscle tissues, devices typically apply dynamic tensile strain [86,87,128,132,133,151–154] or pulsatile hydrostatic pressure [155–158].

Mechanical effects on engineered cartilage tissue

In the case of engineered cartilage a number of studies have shown that mechanical conditioning can enhance chondrogenesis. Importantly, loading parameters, including duration, frequency, and strain rate, have a significant bearing on the responsiveness of chondrocytes and MSCs to loading [131]. Dynamic loading has been shown to increase GAG accumulation and ECM assembly, and, therefore, the mechanical properties of constructs based on bovine calf articular chondrocytes and a variety of 3D scaffolds, including agarose gel [34,146], PGA nonwoven mesh [159], and self-assembling peptide gel [160]. Similar results were also observed for adult canine chondrocytes in an agarose gel under dynamic loading conditions [161]. Application of dynamic loading was also investigated in a layered agarose construct, with encapsulated bovine articular chondrocytes, with varying mechanical properties (2% agarose vs 3% agarose) [162]. These results indicated preferential matrix formation in the 2% agarose layer and an increased elastic modulus in only the initially softer, more permeable layer (2% agarose) [162]. Although the aforementioned studies focused on cells encapsulated in hydrogels, similar results (i.e., increased ECM production and compressive modulus of construct) were found when dynamic loading was applied to calf chondrocytes cultured within a porous calcium polyphosphate scaffold. Overall, the response of chondrocytes to dynamic loading depended on the amount and composition of ECM in the developing construct [163], and in some studies loading increased both synthesis of new GAG and its loss into the culture media [160,164].

The influence of dynamic loading on engineering cartilage has also been investigated in MSC-laden hydrogel systems. In an HA-based hydrogel seeded with human MSCs, dynamic compressive loading enhanced cartilage-specific matrix synthesis and more uniform distribution, increased construct mechanical properties, and suppressed the expression of hypertrophic markers [165]. In another study [166], mechanical loading of MSC-laden agarose constructs prior to chondrogenesis decreased functional maturation and increased chondrogenic gene expression. In contrast, loading initiated after chondrogenesis and matrix elaboration further improved the mechanical properties of engineered constructs, but only when TGF-β3 levels were maintained and under specific loading parameters. Overall, these results demonstrated that the combination of dynamic compressive loading initiated after chondrogenesis and sustained TGF-β exposure may enhance the mechanical properties and matrix distribution of engineered cartilage constructs.

The effects of mechanical stimuli on engineered cartilage may vary among different scaffold systems. In one study, fibrin hydrogels seeded with chondrocytes were cultured under unconfined compression (static and oscillatory) [167]. Compared to the free-swelling control condition, static loading had minimal influence on matrix synthesis or construct stiffness. When comparing the constructs exposed to static versus oscillatory loading, the constructs cultured under dynamic conditions were found

to be softer with less matrix accumulation [167]. Although dynamic compressive loading often results in favorable outcomes in terms of engineering functional cartilage tissue, the scaffold material in which the cells are cultured may influence the effects of mechanical conditioning.

As researchers seek to recapitulate the complex mechanical environment of the joint, the influence of shear, hydrostatic pressure, and tensile forces has also been explored for cartilage regeneration. The application of shear stress is known to stimulate the expression of matrix molecules by chondrocytes [150] and produce constructs with higher amounts of ECM and higher compressive moduli than those exposed to compressive stress [149]. Shear, when combined with dynamic compression, is also a powerful driver of MSC chondrogenesis [168–171]. Likewise, application of dynamic hydrostatic pressure promoted chondrogenesis in 3D cultures of bovine [147] porcine [172] and human [148] chondrocytes. Importantly, cell source strongly influences the effect of loading regime on engineered cartilage constructs. For example, the response of chondrocytes to either oscillatory tensile loading [132] or dynamic compression [173] was shown to be dependent upon the tissue zone of AC (superficial, middle, and deep) from which the cells were isolated. In both aforementioned studies, matrix synthesis stimulated by loading was particularly evident in superficial zone chondrocyte populations. The results of these studies collectively suggest that loading conditions other than compression may enhance the properties of engineered cartilage constructs, and a combination of loading regimens may be necessary to engineer the different zones of AC (reviewed in Klein et al. [53]).

Electromechanical effects on engineered myocardium

In an attempt to bridge the gap between the functional properties of engineered versus native myocardial tissues, the incorporation of electro and/or mechanical stimulation has been investigated by several groups [174,175]. In the case of engineered myocardium, dynamic tensile and pulsatile loading can affect construct composition, contractility, and pharmacological responsiveness [28,86,87,128,132,133,151–153,155–157]. Cyclic stretch affects not only the structure (e.g., orientation of cells and collagen) [152] but also the mechanical function (i.e., contractility) [33,86,87,151] of engineered cardiac tissue. Recently, scaffolds have been specifically designed for mechanical stimulation in cardiac tissue engineering [154]. In this study, chitosan–collagen scaffolds with an array of parallel channels were seeded with rat neonatal heart cells and subjected to dynamic tensile stretch for 6 days using a custom designed bioreactor. Mechanical conditioning promoted cardiomyocyte alignment and elongation and increased cell-to-cell connections as evidenced by increased connexin-43 expression, although these results were dependent on high local stress conditions and were not achieved in areas of the scaffold with lower stress. Instead of using cyclic stretch to provide mechanical stimulation, some studies use pulsatile hydrostatic pressure. One such example used a bioreactor designed to provide physiologically relevant shear stresses and flow rates via pulsatile perfusion [158]. Culture under these pulsatile perfusion conditions enhanced the contractility of the constructs by increasing the contractile amplitude and lowering the excitation threshold.

Excitation–contraction coupling is critical for heart function and reliant upon correct electrical signaling between pacemaker cells via gap junctions. Electrical stimulation of engineered cardiac tissues enhances excitation-coupling by (1) promoting mature differentiation of cardiomyocytes leading to an increase in the number of spontaneously beating cells and (2) synchronizing electrical signal propagation [174]. Radisic et al. were the first to show functional improvement of cardiac tissues engineered using rat cardiomyocytes following continuous electrical stimulation for 5 days (2 ms, 5 V/cm, 1 Hz) [35]. Later studies identified that prolonged periods of electrical stimulation to IPSC-derived cardiomyocyte engineered heart tissues significantly improve sarcomere structure, calcium handling, and contractility [43,88].

In summary, electrical and mechanical conditioning can promote the maturation and functionality of engineered myocardium and is particularly relevant in the case of tissues engineered using differentiated stem cell populations. Such studies also emphasize the potential utility of bioreactors to provide mechanical conditioning in the form of stretch or pulsatile flow in studying and promoting in vitro construct formation.

Conclusion

Engineered tissue replacements must function to meet physiological demands, integrate with host tissue, and have the ability to withstand and adapt to dynamic mechanical and immunological challenges following transplantation. As such, functional tissue engineering requires consideration of multiple endogenous and exogenous variables that when controlled appropriately may work collectively to direct tissue formation. Incorporation of multiple elements (i.e., biomaterials, bioreactors, biological molecules, mechanical conditioning) results in increasingly complex experimental set-ups making comparison between studies challenging. While it would be difficult to standardize in vitro culture parameters, a consensus on outcome measures would enable better comparison between studies as we strive toward a common goal.

Biomaterials can provide structural, biochemical, and mechanical cues, and in combination with bioreactors, growth factors, and mechanical conditioning, these may enhance in vitro generation of functional tissue-engineered constructs. In addition to generating replacement tissues, in vitro tissue/organ models offer a platform from which to test physiological and pharmacological responses in environments mimicking those into which the constructs will eventually be implanted in vivo. Lastly, it is important to note that rapidly evolving technologies in genetics and materials science also may have a significant impact on the future of functional tissue engineering.

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Chapter 11

Principles of bioreactor design for tissue engineering

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Introduction

We review the principles of bioreactor design and its applications in the field of tissue engineering and regenerative medicine. We group bioreactors into two categories: (1) macrobioreactors, which are primarily utilized to grow functional tissues for implantation, and (2) microbioreactors, which are primarily utilized for drug testing, optimization of cell responses under various stimulations and models of disease. In the first part, we describe the principles of design and applications of macrobioreactors, with emphasis on mass transport of nutrients, oxygen, and regulatory molecules into the inner regions of the tissue engineered scaffold and the stimulations of various physiological biomimicry cues, which modulate cell fate and functional tissue assembly. We briefed on bioreactors for sustenance of cell functions in culture and cell manufacturing. In the second part, we describe special design principles for microbioreactors, especially fluidic control in microbioreactors through flow rheology, components, and integration of microfluidic system. We illustrated applications of microbioreactors such as drug testing/screening and models of diseases.

It has been widely established that the oversimplified in vitro two-dimensional (2D) culture models cannot represent the complicated human physiological or pathophysiological responses. Although advancements in three-dimensional (3D) culture systems are promising, the heterogeneous cellular complexity, along with the associated extracellular matrix (ECM) of the native tissue, could not be entirely recapitulated. Over the last few decades, bioreactors offer the engineering solution to recreate the physiological environment of tissue regeneration and are further improved to offer better in vitro and ex vivo culture conditions, to eventually develop into safe, upscalable, and reproducible tissue growth systems.

The most conventional top-down approach to engineering tissues include directing the metabolically active cells into 3D spatial arrangements offered by porous scaffolds, whilst offering the environmental conditions for the growth, differentiation, and maturation of the cells to functional tissues. Macrobioreactors are therefore designed to offer dynamic culture conditions that mimic the native environmental cues such as the biochemical/molecular factors, mechanical and microarchitectural cues. The controllable flow regime of bioreactor offers better oxygen and nutrient diffusion within tissue engineered grafts, which is critical to uniform cell distribution, differentiation, and maturation. Zhang et al. showed the viability of human fetal mesenchymal stem cells (MSCs) in tissue engineered grafts is greatly improved in a biaxial rotating bioreactor (Fig. 11.1) [1] equipped with tumbling and spinning motions mimicking the fetal gyroscopic motion in the mother's womb. Another dynamic bioreactor culture was shown to support stem-cell growth and differentiation uniformly throughout a 200 cm^3 engineered bone graft, the size and shape of the superior half of an adult human femur [2]. Apart from offering dynamic flow conditions for cell viability and differentiation, bioreactors have now evolved to offer biomimicking of human physiological conditions to generate in situ developed organ and tissues for human transplantation [3,4]. The macrobioreactors have found successful applications in developing 3D scaffold–based grafts for both research and clinical translation, recellularization of decellularized tissues, drug screening, and implant testing [5].

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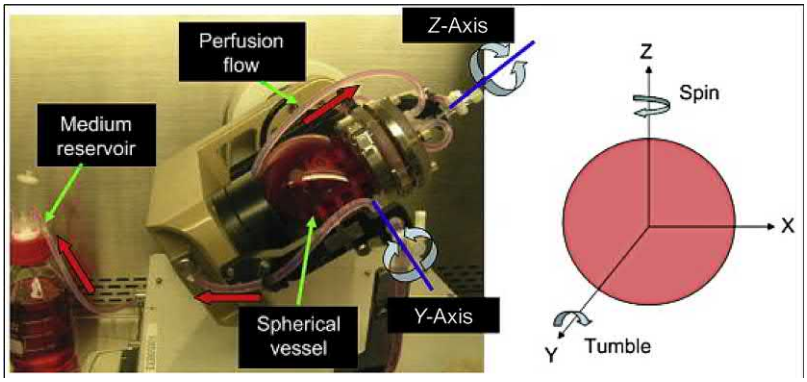


FIGURE 11.1 Biaxial bioreactor design: the bioreactor system consisted of a spherical culture vessel connected to the medium reservoir through tubings through which a perfusion flow is generated. The spherical vessel sits on an articulator which allows rotation in two perpendicular axes (*X* and *Z*). *Modified from Zhang ZY, Teoh SH, Chong WS, Foo TT, Chng YC, Choolani M, et al. A biaxial rotating bioreactor for the culture of fetal mesenchymal stem cells for bone tissue engineering. Biomaterials 2009;30(14):2694–704 with permissions from the publisher.*

While the top-down approach allows flexibility over the choice of materials for the scaffold, such as metals, polymers and ceramics, it does not allow the precise control over microstructure as it relies on the ability of the cells to form the native microstructure and hence not suitable for highly organized tissues with intricate microstructure such as the liver. Although designed to be porous, the scaffolds cannot be penetrated by cells due to diffusional constraints and hence limit cell delivery in large numbers. Another major disadvantage is the poor vascularization of such grafts in vivo, due to the absence of microstructures that facilitate the ingrowth of vasculature [6].

An alternate is the bottom-up approach, which takes advantage of the presence of repeating structural and functional units in tissues/organ. Bottom-up tissue engineering involves generating modules, composites of cells and the matrix, which are assembled to generate the graft. This approach allows precise control over the microstructure and is particularly advantageous when multiple types of cells are required to recreate the functional tissue. The modular assembly also ensures uniform cell loading throughout the graft and allows the accommodation of structures like channels that can facilitate vasculature. Several methods of module generation such as 3D cell–laden hydrogels using micromolding, photolithography and cell sheets, and assembly techniques such as photopatterning, random assembly, flow-directed assembly using microfluidics and random packing have been employed to create functional tissues [7]. Newer method of rapid prototyping–based 3D bioprinting has been extensively used in modular tissue engineering [8]. Scaffold-free approach involves the printing of cells in an ECM-based hydrogel matrix and allows precise microstructural control. This method allows the use of multiple cell types, which have been exploited to form endothelial cell–laden vasculature that allows vascularization and improved integration into the host tissue. Precise microstructural control that enables native tissue-like microstructure, incorporation of multiple cells types (parenchymal and mesenchymal cells), and the incorporation of vasculature have allowed the successful generation of functional tissues for bone [9], cornea [10], liver [11], skin [12], kidney [13], and heart valves [14].

Macrobioreactors

In 1994 in the United Stated alone, more than 18,000 organ transplants were performed with almost double the number of patients on the waiting list for a life-saving procedure [15]. Fast forward to 2017, there have been more than 34,000 organ transplants performed with more than 115,000 patients on the waiting list [15]. To meet this great demand, new strategies are needed to restore these damaged tissues. Tissue engineered substitutes generated in vitro could be the alternative to this. These substitutes should have organ-specific properties with respect to biochemical activity, microstructure, mechanical integrity, and biostability [16]. The generation of 3D tissue substitutes in vitro requires not only a biological model but also the development of new culture strategies such as the bioreactors [17,18].

The first bioreactor was developed in 1857 [19], but it has only been widely used in tissue engineering and regenerative medicine until the concept of tissue engineering was formally proposed and defined by the National Science Foundation (NSF) (United States) in 1987 [20]. The core of tissue engineering is the application of cell biology and engineering principles to develop biologically active human tissue substitute for restoration of tissue architecture and function after an injury or disease. The application of tissue engineering to construct engineered tissue is achieved mainly through the growth of cells in a variety of scaffold in vitro, later combined with certain kinds of cytokines, to promote the lineage commitment of cells and the formation of desired tissues or organs. Three key elements of tissue engineering: cells, scaffold, and cytokines are involved [16]. However, the surrounding environment in which the three elements interact in vitro is different from that in vivo, and the

three elements are usually combined, lacking the synchronization with various environmental factors such as mechanical stress and electric field.

Abundant evidence showed that the efficiency of substance exchange between engineered tissue and culture medium, the density and spatial distribution of cells inside, and the various environmental factors that the cells are exposed to play an important role in the development of engineered tissue. For example, due to the impedance of mass transfer, the "dead zone" phenomenon of cell necrosis occurs in the centrum of engineered tissue [1], and the maximum size of engineered tissue is greatly limited accordingly [21]. By the traditional manual cell seeding method, not only the cell load density is difficult to match the demand of the tissue substitute but also the cells are not evenly distributed uniformly in the tissue, which directly affects the quality of the engineered tissue. Tissue-engineering bioreactor is developed to mitigate above challenges, including large-scale cell manufacturing, massive and homogeneous cell growth in bulky tissue, high efficient supply of nutrients, and the removal of metabolites as well as essential environmental stimulations, imposed on cells [17]. In Table 11.1, we listed various commercial macrobioreactors with their advantages and disadvantages [22]. On the one hand, it can become an important means to investigate the modulations of different environmental stimulations on the 3D functional culture of specific cells and tissues. On the other hand, it is one of the keys to improve the quality of functional tissue and reduce production cost. Moreover, it is also a key step in the translation of output of tissue engineering from bench to industry for large-scale production and implementation of standard operating guidelines and procedures.

Design principles

Mass transport

Tissue-engineering methods are a promising strategy for overcoming the lack of organs for transplantation. However, its introduction into the clinical setting is mostly limited to thin tissues such as skin. One main clinical challenge hampering the widespread adoption of tissue-engineering strategies is the poor cell survival, particularly at the core of thick voluminous grafts [23]. Growth of thick cellular grafts in a static culture environment is challenged by mass transport (of oxygen, nutrients, waste products, and metabolites) that solely depends on passive diffusion within a cellular graft, which is often limited to short distances (100−200 μm), leading to inadequate nutrient supply that is required for cell survival [24].

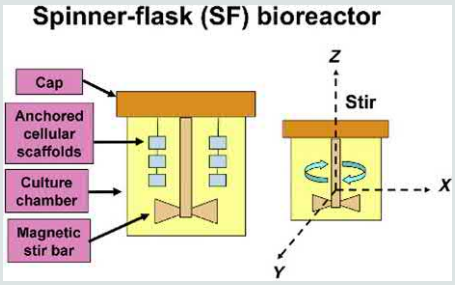
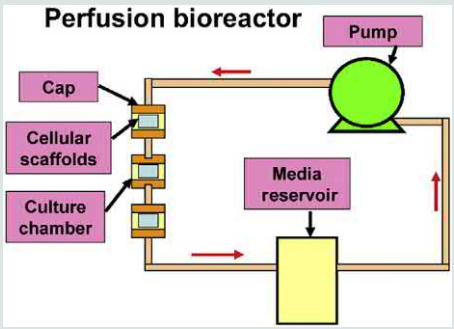
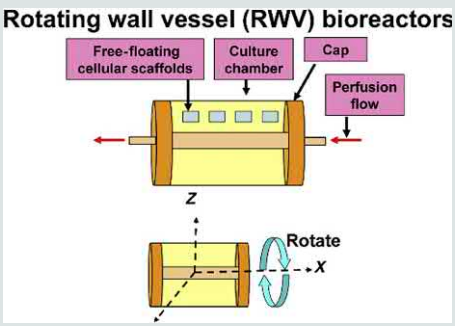
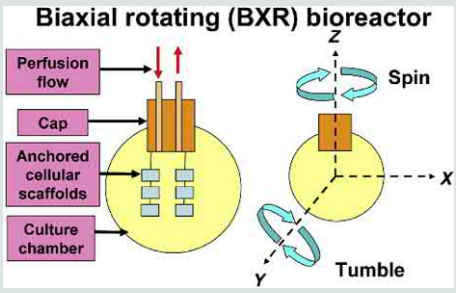
To promote cell survival, bioreactors must provide adequate mass transport of nutrients, oxygen, and biochemical factors to the cells. They have to be efficiently transported from the bulk culture medium to the tissue surfaces (external mass transfer) and then through the tissue to the cells (internal mass transfer). Similarly, metabolites and CO_2 are removed from the cells through the tissue matrix to the surfaces and then to the bulk medium. In physiological settings, these functions are provided by the vasculature, which readily adapts to the changes in metabolic demands of the cells. Therefore consideration of mass transport of nutrients, oxygen, and regulatory molecules is essential for bioreactor design.

A constant supply of nutrients to cells is crucial for the development and growth of engineered tissues. In the case of primary and/or stem-cell cultures, the delivery of nutrients is essential to induce the differentiation and expression of a desired cellular phenotype. However, in tissues with clinically relevant sizes, the mass transport of nutrients into the engineered tissue is often insufficient to support the growth and development of all the cells within the tissue [25]. This may lead to a concentration gradient of nutrients with high concentrations at the surface and low concentrations in the center of the tissue [26]. Cells at the periphery will experience different environmental conditions compared to cells located in the center and may eventually lead to tissue necrosis in the central regions and nonhomogeneous tissue development throughout the construct [27−29].

In the human body, almost every cell is close to a blood vessel that provides nutrients for its survival. But cells in tissue engineered scaffolds where a vascular network is absent may only receive nutrients via diffusion, which is a slow process and over distances limited nutrients may result in poor cell proliferation or nonoptimal conditions for new matrix production [30]. An obvious strategy is to enhance vascularization of tissue engineered constructs, and this may be achieved by inclusion of angiogenic factors and through scaffold design. However, it must be noted that such approaches still ultimately are dependent on the mass transport of nutrients which in turn influences the rate of vascularization, which would take multiple days to weeks before the center of the implant becomes perfused [31,32].

In the field of tissue engineering the supply of nutrients is critical yet often overlooked [25]. Research in this field has focused to address these concerns, and the development of bioreactors is a promising approach to address the availability of reduced nutrients to the inner regions of the tissue engineered scaffold. In bioreactor systems, perfusion of the culture medium through the engineered tissue was widely utilized. The result is that nutrients are no longer only transported to the cells within the tissue by means of diffusion but also by convection. From Eq. (11.1) the contribution of perfusion to nutrient

TABLE 11.1 Table of comparison of commercial macrobioreactors.

Types of bioreactors	Advantages	Disadvantages
	Improved mixing within the media Low cost	Turbulence generated by the impeller can be detrimental for seeded cells and newly laid down extracellular matrix
	Low shear forces and high mass transfer	Nonhomogenous cellular distribution Cells at frontal zones are washed away by the oncoming perfusion flow with higher flow rates
	Low shear forces and high mass transfer (similar to perfusion bioreactors)	Nonhomogenous cellular distribution and extracellular matrix deposition In the free-floating culture, collision between scaffolds and bioreactor walls may induce cellular damage and disrupt cellular attachment and matrix deposition on the scaffolds
	Physiologic biomimicking of fetal biaxial rotation and microgravity experience. Shear forces and mass transfer controllable. Homogenous cellular population in scaffolds. Amenable to automation	Occupy more space and weigh heavier due to the biaxial fixtures for rotation

Source: Modified from Zhang ZY, Teoh SH, Teo EY, Khoon Chong MS, Shin CW, Tien FT, et al. A comparison of bioreactors for culture of fetal mesenchymal stem cells for bone tissue engineering. Biomaterials 2010;31(33):8684–95 with permission of the publisher.

transport is directly related to the velocity of the medium. In order for the contribution of perfusion to nutrient transport to become significant, the velocity should be high enough so that the Péclet number becomes larger than 1 (see Eq. 11.2) [25].

$$J_a = -D_a \cdot \frac{dc_a}{dx} + u \cdot c_a \qquad (11.1)$$

where J_a is the flux of component a (mol/m^2/s), D_a is the diffusion coefficient of component a in the medium (m^2/s), c_a is the concentration of component a (mol/m^3),

x means the distance (m) [33], and u is the bulk convective velocity of the medium (m/s).

Whether nutrient transport is dominated by diffusion or convection depends on the relation between the diffusion coefficient of the nutrient (D) and the flow speed of the medium (u). This relation between the contribution of diffusion and convection to the overall transport is described in the dimensionless Péclet number.

$$Pe = \frac{L \cdot u}{D} \tag{11.2}$$

where L is the characteristic length (m) (the distance over which diffusion takes place), D is the diffusion coefficient of the nutrient in medium (m^2/s), and u is the bulk convective velocity of the medium (m/s).

Tissue-engineering studies under 2D and/or static conditions has proven extremely difficult to promote the high-density 3D in vitro growth of cells that have been removed from the body and deprived of their normal in vivo vascular sources of gas exchange [34]. Delivering sufficient oxygen supply in 3D tissue constructs poses a challenge for tissue-engineering applications as the thickness and structure of the scaffold affect oxygen diffusion, which is essential for the survival of cells seeded within the scaffold [35,36]. The diffusion limit of oxygen in dense tissues, such as the skeletal muscle or bone, is less than 200 μm^2 [21,36,37] and poses a major problem for tissue-engineered cell constructs, since cells in the inner part of the scaffold are in danger of oxygen deprivation, which affects cellular functions of metabolism and differentiation for example [38].

It must be noted that oxygen concentration in air is about 21% while the physiological oxygen levels in the blood range between 10% and 13% [39]. Furthermore, oxygen levels vary depending on the tissue type and location. For example, oxygen levels in highly vascularized tissues such as bone and bone marrow are $\sim$5%−10% and $\sim$2%−7%, respectively, and is in contrast to avascular tissues such as cartilage $\sim$1%−6% [40,41].

The flow regime generated within bioreactors helps to overcome the limitations of oxygen diffusion within tissue-engineered grafts, which is critical in maintaining cellular survival within the graft and uniform cellular distribution [5]. The selection of the exact conditions of bioreactor cultivation that provide the necessary level of oxygen transport is in both cases supported by the use of mathematical models of oxygen supply and consumption within the cultured tissue. Tissue-engineering bioreactors seek to improve external mass transfer of oxygen. However, as each tissue has its own unique functions and characteristics, it should not be a one-size-fits-all approach.

Controlled delivery of bioregulatory molecules in cell cultures is essential to induce the expression of a desired cellular phenotype. Molecular transport is dominated by diffusion (an exception being transport through motor protein such as kinesin and dynein), while cell behavior is influenced by cell−cell and cell−matrix interactions, and cell-signaling pathways rely on the presence of promoters and inhibitors and their affinities for the receptors [42]. Although many of these molecules have been identified, the ability to specifically control cell behavior through multiple regulatory pathways remains a challenge. The capability to modulate regulatory factors can lead to the recreation of native-like environments and allow the quantification of cell−cell interaction, and the use of bioreactors together with innovative design can provide a solution to optimize cellular response.

In stem/primary cells for example the cell fate and tissue assembly during early development and tissue remodeling are regulated by multiple cues acting across over time. During development and regeneration, tissues are formed from coordinated sequences of stem-cell renewal, specialization, and assembly that are orchestrated by cascades of regulatory factors. Growth media provides the necessary nutrients for cell proliferation and/or differentiation, while metabolites are continuously released into the media. The ability to systematically ascertain specific culture conditions within the bioreactor vessel and subsequently regulate a feedback response is essential for achieving effective tissue growth [5], and the challenge is to accurately recapitulate this in a bioreactor by providing an environment where this can occur.

Computational fluid dynamic techniques can be used to design the optimal microenvironment for culture of cell-laden porous scaffolds in perfusion bioreactor. Such an example would be that of adherent bone marrow stem cells and nonadherent hematopoietic stem cells that predict the effects of microchannel-provided scaffolds with different geometries on fluid-dynamics and oxygen transport [43]. Advantages of computational simulations include the ability to modify and study the effects of bioreactor design with respect to the flow analysis, without having to develop and construct actual physical models, or to run a large number of experiments. This is coupled with the significant savings in time and costs. Furthermore, visualization of flow as enabled by the simulation package is a key factor in determining the efficacy of the system and allows for design optimization prior to bioreactor design and modifications [34]. In general, these simulations assist in identifying critical issues and problems, for example, in approximating the locations of recirculation zones. These zones may potentially damage cells and inhibit growth. Furthermore, these recirculating bodies may impede the flow of fluid into and out of the scaffold. The choice of flow regime is therefore of great importance.

Taken together, the mass transport of nutrients, oxygen, and regulatory molecules is critical to the growth and

survival of the tissue. Recent advances in bioreactor design have focused on mimicking the native environments and thus providing efficient mass transfer to growing tissue. Nevertheless, this is just one aspect of bioreactor design and the considerations of various stimulations and cell environment will be discussed below.

Physiological biomimicry cues

From the advanced knowledge of cell and developmental biology, we realize that cell as the seed of developing tissue and organ will remodel its microenvironment and also integrate into a functional whole orchestrated by surrounding hierarchal cascades of regulatory factors in vivo, temporally and spatially [44]. Therefore to construct a specific bioactive and functional engineered tissues in vitro, it is essential to mimic the native physiology of the tissue [40] such as shear stresses/hydrostatic pressure of a native blood vessel or the compression/torsion of the native cartilage [41]. In this section, amongst the various environmental stimuli equipped in bioreactors, we would like to highlight the role played by mechanical, electrical, and magnetic stimulations for tissue development in bioreactors (Fig. 11.2).

It is widely accepted that mechanical stress stimulation is an important factor regulating the physiological functions of cells and plays an important role in promoting the formation of specific tissue, especially in musculoskeletal tissue, cartilage tissue, and cardiovascular tissue. Based on the hypothesis that biomimicry of physiological mechanical loading will potentiate accelerated osteogenesis, Ravichandran et al. [45] demonstrated the application of physiological cyclic compressive loads on MSCs-laden 3D polycaprolactone-β-tricalcium phosphate scaffolds in bioreactor with physiological cyclic strain (1 Hz) of 0.22%. Results showed the enhancement of expression of osteogenesis-related genes (osteonectin and collagen type I) on week 1 and activity of alkaline phosphatase on week 2 after compressive loads of compressive groups on comparison with their static controls, respectively. Moreover, mineralization of compressive group was found to approach saturation on week 2 after compressive loads. The results provided a proof that biomimicry of physiological mechanical cues is essential for the maturation of engineered bone grafts cultured in vitro.

Maturation of cardiomyocytes differentiated from stem cells is important for regeneration of functional cardiovascular tissue. Ruan et al. investigated the effects of exogenous cyclic tensile stress on myocardial differentiation and maturation of human induced pluripotent stem (iPS) cell, exposed to uniaxial cyclic tensile strain with a frequency of 1 Hz, 5% elongation generated in FX-400T bioreactor [46]. By flow cytometric analysis, it was observed that cyclic tensile stress stimulation increased cTnT intensity by about 1.3- to 1.5-fold over their controls, suggesting that cyclic stress does have a significant effect on cardiomyocyte maturation. In addition, cyclic stress stimulation also increased the expression of β-MHC versus their controls. Concurrently, α-MHC gene expression significantly decreased with cyclic stress stimulation as compared to their controls, which corresponds to the increasing human gestational age.

Bioreactors equipped with torsional stress stimulation had been developed to study the formation of engineered ligament tissues and intervertebral discs [47,48]. Scaglione et al. [47] designed a compact bioreactor system to apply automated cell-culturing procedures onto engineered tissue under controlled torsion/traction regimes to recapitulate ligament mechanical environment in vivo. Fibroblast-laden poly-caprolactone-based scaffolds were exposed to a torsional stimuli consisted of forward/back-forward sequential cycles of 100 degrees from neutral position at a rate of 600 degrees/min. After 3 days

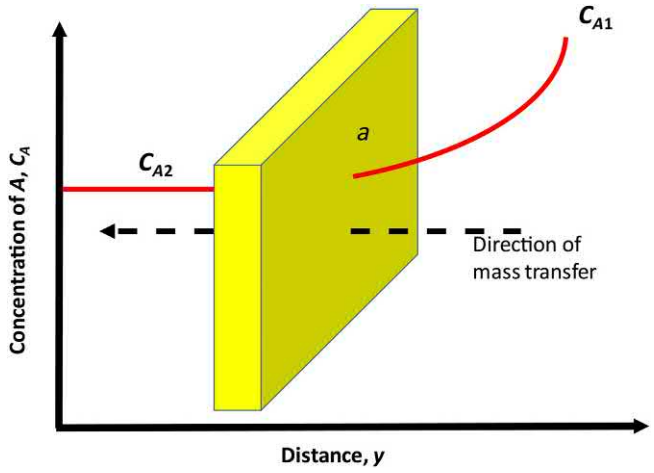


FIGURE 11.2 The concentration gradient of component "A" in a stagnant system (no convection) inducing mass transfer across area "a." The diffusion occurs in the direction required to destroy the concentration gradient of the molecular components "A." The concentration of "A," varies as a function of the distance y and $C_{A2} > C_{A1}$. *Adapted from Doran et al. (2013) with permission of Rouwkema J, Rivron NC, van Blitterswijk CA. Vascularization in tissue engineering. Trends Biotechnol 2008;26(8):434–41.*

of stimulation the cell morphology was partially changed with preferential orientation of cell actin filaments along the torsional direction as opposed to the random orientation of actin fibers in their controls. In addition, the mRNA expressions levels of collagen type I, tenascin, and collagen type III were 2-, 6-, and 31-fold higher in simulation groups compare to their controls, respectively.

To mimic the shear stress condition induced by blood flow in the human body such as the physiological interstitial flow over osteocytes during bone compression and the blood flow through arteries and veins, bioreactors applying shear stress stimulation were developed to improve the maturation and development of engineered tissues [49–51]. van Haaften et al. [50] developed a novel bioreactor that decoupled the effects of two types of hemodynamic loads: the shear stress and the cyclic stretch for developing vascular grafts. Two independent pumps were used to perfuse medium and to distend the electrospun polycaprolactone bisurea scaffold in a computationally optimized culture chamber. There was no preferential orientation in actin fiber direction for statically cultured samples, whereas a clear preferred actin fiber orientation was observed in samples exposed to shear stress or cyclically stretched samples. Moreover, the contribution of shear stress (1 Pa) overruled stretch (1.05 Pa at 0.5 Hz)-prompted cell proliferation (DNA/tissue) and ECM production (GAG/tissue).

Electrical signals are known to be one of important regulators of embryonic development on stem-cell division and differentiation and also play a critical role in healing wounds as a mediator to direct cell migration into the wound [52,53]. The integration of electrical stimuli in the bioreactor design resulted in improvement of the structure and functionality of engineered cardiac tissues [54]. Balint et al. (2013) reviewed the importance of endogenous electrical stimulation, methods of delivering, and its cellular and tissue-level effects in the engineering of nerves, cardiac and skeletal muscle. Visone et al. [52] developed a novel oscillating perfusion bioreactor (Fig. 11.3) in which neonatal rat cardiac fibroblasts–laden collagen tissue were cultured with a combination of bidirectional interstitial perfusion and biomimetic electrical stimulations. Result showed that electrical cues reduced the excitation threshold from

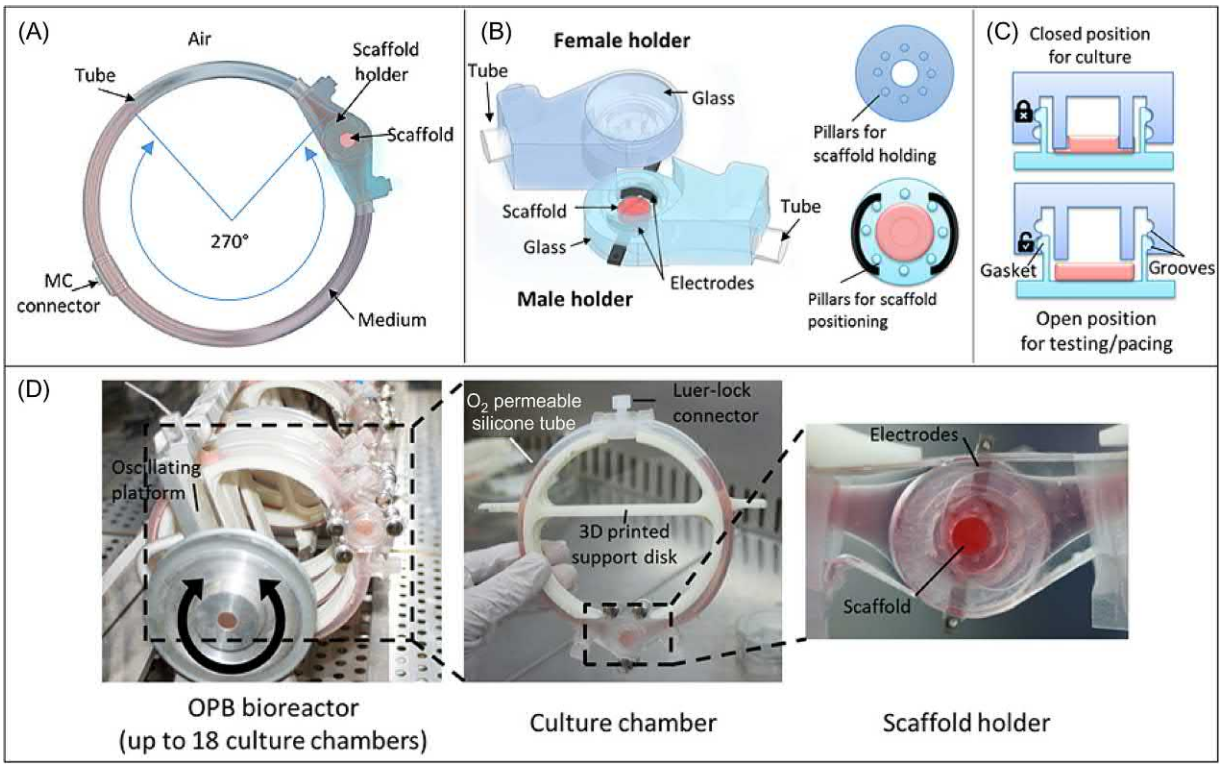


FIGURE 11.3 (A) Scheme of the bioreactor chamber including the scaffold holder and the connector; medium perfusion through the scaffold is achieved by bidirectional oscillation. (B) Scheme of the scaffold holder composed by two complementary parts housing electrodes, two round arrays of pillars, and glass windows. (C) Representation of the two functioning positions of the scaffold holder to hold the construct during culture (top) and to release it for testing (bottom). (D) The bioreactor housing the PDMS culture chambers mounted on 3D printed supporting disks. *Adapted from Visone R, Talò G, Lopa S, Rasponi M, Moretti M. Enhancing all-in-one bioreactors by combining interstitial perfusion, electrical stimulation, on-line monitoring and testing within a single chamber for cardiac constructs. Sci Rep 2018;8(1):16944 with permissions from Springer Nature Publishing AG, https://doi.org/10.1063/1.5037968.*

5.2 ± 0.95 V in perfusion only group to 4.35 ± 0.8 and 4.2 ± 0.5 V in electrical stimulation only group, perfusion and electrical stimulations group, respectively. Moreover, the gene expression of MYH6, indicative of immature cardiac phenotype, decreased only under combination of perfusion and electric stimulations condition, leading to an increased MYH7/MYH6 ratio versus their perfusion controls, implying a more adult-like cardiomyocyte phenotype achieved under the combination of stimulations.

Considerable research reports (Table 11.2) documented that magnetic field–specific pulsed electromagnetic field (PEMF) influences osteoblast and MSCs proliferation and differentiation through initiating several signaling cascades such as Ca^{2+}, Wnt/β-catenin, mTOR, and some growth factors [55–71]. Bassett et al. (1978) successfully used PEMF to treat nonunion fractures, and PEMF was approved as a noninvasive method for treating delayed union or nonunion fractures by the US Food and Drug Administration in 1979. Currently, PEMF has been used for bone and cartilage-related pathologies, including knee and cervical spine osteoarthritis [60,61]. Even though PEMF is still in its infancy, it holds great potential as a form of prospective, noninvasive treatment.

Fassina et al. designed a simple electromagnetic bioreactor (magnetic field intensity, 2 mT; frequency, 75 Hz) to stimulate osteosarcoma cells cultured in a 3D polyurethane scaffolds [69]. Results showed a high proliferation rate (approximately twofold), more matrix proteins production on scaffold surfaces, and more calcium deposition (approximately fivefold) in the electromagnetic stimulation groups as compared to their no stimulation controls. Consistently, RT-PCR analysis revealed the electromagnetically upregulated transcription of matrix proteins [decorin, fibronectin, osteocalcin, osteopontin, transforming growth factor-β (TGF-β), type I collagen, and type III collagen].

Recently, Mayer-Wagner et al. developed a novel bioreactor in where low-frequency electromagnetic fields (LF-EMF) and simulated microgravity (SMG) stimulations were applied to investigate chondrogenesis of MSCs [72]. The LF-EMF (sinusoidal, 15 Hz and 5 mT magnetic flux density) was applied three times a day for 45 minutes throughout the differentiation period of 21 days. MSCs pellets exposed to SMG and LF-EMF showed strong positive signals in both safranin-O and collagen type II staining compare to SMG alone and LF-EMF alone controls. Moreover, RT-PCR analysis revealed that LF-EMF/SMG induced a significantly higher COL2A1 expression compared to the SMG alone control but did not reach the no stimulation control level. Results demonstrated that combination of LF-EMF and SMG stimulations provided a rescue effect of the chondrogenic potential of MSCs, while LF-EMF stimulation alone did not interrupt any gene expression changes of MSCs.

Cell environment

Understanding, recreating, and controlling the overall native complex cell environment are a key to engineering tissues via bioreactors. Supplementing scaffolds that mimic the ECM, providing biophysical environment of the native tissue and modulating the spatial and temporal gradients of the regulatory factors, are some of the well-known methods to fabricate the cellular environment in macrobioreactor technologies.

3D scaffolds are used in combination with bioreactors not only as a substratum for cell growth but also for enhanced nutrient flow and oxygen diffusion to prevent formation of necrotic zones. Naturally occurring ECM carries inductive signals for phenotype determination of the cell, as well as vital for cell adhesion and growth [73]. Tissue engineered scaffold—corresponding to the natural ECM—are used in the bioreactors as a template for tissue regeneration. Decellularized ECMs that are derived from the native whole tissues are often considered the gold-standard of scaffold constructs. Decellularized matrix offers the native cell environment with the preserved micro- and microscale structural and functional ECM proteins that provides the necessary cues for cell adhesion, proliferation, and maturation. However, major challenges in employing the decellularized matrix for bioreactor technologies include the difficulty in sterilizing them without damaging the structural and mechanical properties and the host immunogenicity of the degradation products [74].

Synthetic scaffolds are being widely employed as tissue-engineering scaffolds for bioreactors, which offer advantages such as uniformity in batches, lack of host immunogenicity, and the ease of sterilization and further clinical translation. Main considerations of scaffold design include the size, geometry, porosity, density of pores, the windows connecting the pores, and the surface properties [75]. Either synthetic or natural polymers are chosen to fabricate the scaffolds. While synthetic polymers are more stable with longer shelf-life and can be readily sterilized, natural derivatives offer bioactive properties, with good biocompatibility and reduced toxicity [76]. Altering the composition, structure, and arrangement of the synthetic polymer scaffold constituents offer a wide range of properties such as porosity as well as tailored pore sizes, rate of biodegradation, and mechanical properties.

A wide array of scaffold fabrication techniques such as solvent casting/particulate leaching, melt moulding, freeze–drying thermally induced phase separation, gas foaming, 3D printing, and electrospinning are being employed to achieve a wide variables of scaffold architecture and design. Each approach has its own advantages and disadvantages, where, 3D printing and

TABLE 11.2 Research studies that applied magnetic field to influence cell proliferation and differentiation.

Magnetic field	Cells	Effect	Mechanism proposed	Ref.
PEMF, 3 ms, 50 Hz, 0.6 mT	MC3T3-E1	Prompted cell proliferation	PEMF regulates intracellular calcium ions	[57]
PEMF, 1.3 ms, 75 Hz, 1.5 mT	MSCs	Increased expression of osteogenic markers and transcription factors investigated	PEMF modulates Notch genes involved in osteogenesis	[62]
PEMF (from MED, Magdent)	MSCs	Increased cell proliferation and adhesion and the osteogenic commitment	PEMFs activated the mTOR pathway to stimulate osteogenic differentiation	[61]
PEMF, 67 ms, 15 Hz	Bone marrow macrophages	Inhibited osteoclast formation	Osteoblastic genes were stimulated by PEMF, including genes that inhibit osteoclastogenesis	[60]
PEMF, 6 ms, 15 Hz, 2 mT	MSCs	Prompted chondrogenic differentiation	PEMF-regulated calcium entry through transient receptor potential channels	[58]
PEMF, 5 ms, 15 Hz, 2 mT	MC3T3-E1	Enhanced cellular attachment, proliferation, and osteogenic differentiation in vitro Promoted osteogenesis, bone ingrowth, and bone formation in vivo	PEMF promoted skeletal anabolic activities through a Wnt/β-catenin signaling	[56]
PEMF, 1.3 ms, 75 Hz, 2 mT	MSCs	Prompted osteogenesis	PEMF enhanced intracellular calcium concentration	[63]
PEMF, 5 ms, 15 Hz, 0.96 mT	Osteoblasts	Increased protein adsorption and enhanced the initial osteoblast adhesion Accelerated osteoblast proliferation and differentiation	PEMF changed the electric potential gradients of the surface and induced surface polarization on the cell membrane	[64]
PEMF, 6 ms, 20–50 Hz, 2–5 mT	MCF-7 and MCF-10	Increased MCF-7 damage after PEMF exposure. No effect on MCF-10	Difference in intracellular calcium handling of Ca^{2+} in response to PEMF exposure	[65]
ELF-EMF, 18 Hz, 2.5 μT	Cardiac stem cell	Increased in the expression of cardiac markers	Intracellular Ca^{2+} accumulation and mobilization	[66]
PEMF, 48 Hz, 1.55 mT	MC3T3-E1 Osteoblasts	PEMF influenced proliferation and differentiation of osteoblast but not MC-3T3	Osteoblasts are more sensitive to PEMF compared with immortalized cells	[67]
PEMF, 300 μS, 7.5 Hz, 0.13 mT	Osteoblastic cells	Speed up apoptosis of osteoclasts	PEMF elevates the intracellular and nucleus Ca^{2+} concentration and increases endonuclease activity	[68]
PEMF, 1.3 ms, 75 Hz, 2 mT	Osteosarcoma cell line SAOS-2	Improved cell proliferation Increased gene and protein expression of osteogenic biomarkers Increased calcium deposition comparing with static culture	PEMF increases the cytosolic Ca^{2+} concentration	[69]
Sinusoidal MF, 50 Hz, 0.8 mT	ES-derived cardiomyocytesEmbryoid bodies	Increased the expression of the cardiac specific transcripts α-myosin heavy chain and myosin light chain	Coupling of MF with GATA-4, Nkx-2.5, and prodynorphin gene expression may represent a mechanism pertaining to ES cell cardiogenesis	[70]
PEMF, 300 μS, 7.5 Hz, 0.13, 0.24, and 0.32 mT	Bone marrow cells	Regulated the formation of osteoclast-like cells in bone marrow culture in magnetic field intensity–dependent manner	Correlations between osteoclast-like cells formation and cytokines depending on the induced electric field intensities	[71]

MSC, Mesenchymal stem cell; *PEMF*, pulsed electromagnetic field.

electrospinning are the widely used techniques [73]. Porous fabricated scaffolds are seeded with suitable cells and grown in a wide range of bioreactors, such as spinner flasks, rotating wall vessels (RWV), and perfusion bioreactors, to obtain the functional engineered tissue succedaneum.

The scaffolds or the substratum for the cells in culture for bioreactors are usually enriched with biological factors for cell fate determination. The biological factors required for the bioreactor-scaffold system depends on specific application of the scaffolds. For example, human embryonic stem cells were known to exhibit lesser cell differentiation, with more tendency toward self-propagation with pluripotency, when they were cultured with laminin 521 [77]. While culturing the neural stem cells in an injectable hydrogel of silk fibroin, IKVAV short peptide was observed to effectively improve the activity and differentiation of the cells [78]. For vascular tissue engineering of arteries, treatment with TGF-β, sphingosylphosphorylcholine, and bone morphogenetic protein-4 is required along with mechanical stimulation of the adipose derived stem cells in pulsatile perfusion systems [79]. Growth factors such as bFGF and EGF are known to promote the proliferation and controllable migration of stem cells. In neural cell culture, nerve growth factor and brain-derived neurotropic factor enhance the survival of cortical neurons and proliferation [80]. In cartilage tissue engineering, addition of growth factors TGF-β1 or IGF-I was known to greatly improve the mechanical properties of tissue engineered constructs of chondrocyte-seeded agarose hydrogels under dynamic deformational loading [81]. Overall, a plethora of biological factors can be used with the scaffolds and bioreactors to successfully engineer tissue constructs in vitro.

Controlled modification the biological and/or physical properties of the scaffolds is vital in guiding the cell migration, adhesion, growth, and differentiation [82,83]. Mimicking complexity of the native protein family in ECM is achieved by patterns of adhesive and nonfouling proteins, which further influences the spatial distribution of cells and formation of focal adhesions. In addition to the cell substrate, cell shape plays an important role in determining the cell fate and function [84]. Geometrical cues at micro and nanoscale therefore has varied responses on different cultured cells. Substrate stiffness is known to play a vital role in lineage specification of stem cells. For example, altering the elasticity of the polyacraylamide gels with coated collagen I or other adhesion proteins for cell attachment to mimic the in vivo–like matrix elasticity, soft matrices were observed to be neurogenic, stiffer ones were myogenic, and the more rigid ones were osteogenic [85–87]. Combination of mechanical and biochemical properties of the cell culture substrate enabled the study of plethora of cell environment suitable for specific tissue culture responses. Patterning techniques help to precisely control the microenvironmental assembly of mechanical and biochemical stimuli of the substrate. Few of the patterning techniques include

1. microcontact printing of alkanethiols and proteins on suitable substrates,
2. replica molding for fabrication of micro and nanostructures in polyurethane or epoxy, and
3. solvent-assisted micromolding of nanostructures in poly(methyl methacrylate).

Another key player in cell fate determination in bioreactors is the interaction with other cell types. The possibility of compartmentalization of cells in bioreactors provides a suitable platform for multiple population cocultures that are otherwise challenging to establish in vitro [88]. For example, MSCs are cocultured with endothelial cells for creating simultaneous calcification and vasculature of bone tissue–engineering scaffolds [89].

Sustainable bioreactors

Among the various design considerations for bioreactors, long-term sustenance of cell culture and flexibility over scalability is critical for allowing the translation of the technology to a clinically relevant setting. The scaling up of bioreactors is technically challenging because of the loss of microenvironmental control in large-scale processes, which impair cell functionality. For example, the increase in the shear stresses due to hydrodynamic forces required to achieve optimal mass transport and oxygen diffusion throughout the bioreactor might damage the cell membrane and the lead to loss of cell viability and functions. The trade-off, between the operating process parameters required to sustain large numbers of cells for long periods, and the control of the cellular microenvironment that affects the cell functions, has to be carefully considered for the cell type and the intended application.

An example for an effective bioreactor design, which integrates the microenvironmental culture parameters while allowing efficient scale up for functional hepatocytes, has been demonstrated by Xia et al. [90]. The primary hepatocytes were cultured in between layers of collagen. This sandwich collagen culture served two purposes: it provided the matrix cues which allowed the primary cells to regain polarity that is critical for the functioning of the hepatocytes and protected the hepatocytes from the shear by shielding the cells from direct exposure to flow. A total of 12 sandwiches were stacked and assembled into the bioreactor with holders and slits, the configuration allowed the serial flow of the culture medium without bypass. With a flow of 1 mL/min, the hepatocytes maintained viability and functionality in

terms of metabolic and transporter activities, and albumin and urea production in the bioreactor for 7 days. This was a significant improvement over the existing systems that did not support the survival and functionality beyond 3 days in bioartificial liver applications. Also the authors have proposed a clear strategy to scale up the existing model for use in clinics without drastic modification of the plate dimensions, which would have required recalibration of the fluid dynamics and with minimal priming volume. This is an ingenious example of an efficient bioreactor design that provides adequate microenvironmental control to preserve cell functions and allows for the sustenance of the large numbers of cells over long periods of time. Such strategies could be adopted to improve the efficacy, scale-up, and the sustenance of bioreactors for other cell types and applications.

Cell manufacturing quality attributes and process analytics technology

The advances in tissue engineering aimed to develop prosthesis, extracorporeal assist devices, tissue grafts, and whole organs necessitate optimal cell manufacturing processes to ensure the production of safe and potent cells suited for the desired application in clinically relevant numbers. Optimal cell manufacturing processes integrate several critical steps such as identification of legitimate sources of cells, isolation and purification of the appropriate cell types, large-scale expansion, storage, and retrieval of cells, all of which in compliance with the regulations of statutory boards. Cell manufacturing involves the large-scale expansion of cells in clinically relevant numbers without compromising the quality of the cells in terms of the efficacy and safety. Efficacy refers to the ability of the cells to perform the desired functions, while the safety concerns the undesired side effects of the cells when used in vivo. For instance, loss of the enzymatic function of hepatocytes on expansion compromises the efficacy; the activation of CAR T cells toward autoantigens while expansion critically compromises the safety. In addition to safety and efficacy the cell manufacturing process should include the control of the variability from batch to batch to reliably assess the performance in therapeutic applications.

Hence, the cell manufacturing processes must carefully consider the bioreactor design, nominate the critical quality attributes (CQA) and critical process parameters (CPP), and implement real-time monitoring, all based on the cell types and the applications. The CQA are the biological, physical, and biochemical attributes that can reliably predict the viability and functionality of the cells for the specific application and analyze the variability in the cell populations. The CPP are the operating parameters such as temperature, oxygen diffusion rate, shear stress, and pH, which can impact the CQA. The CPP have to be monitored and analyzed real time to provide feedback to avoid impairments of the CQA. The bioreactor design has to be optimized to preserve cell functionality and allow scalability. Taken together, the formulation of a cost-effective and an efficient cell-manufacturing pipeline requires a multidisciplinary approach harnessing the technical prowess of clinicians, biologists, chemical and process engineers, and data analysts. The National Cell Manufacturing Consortium is one such initiative by the NSF Cell Manufacturing Technologies involving collaborative efforts of hospitals, academic institutions, pharmaceutical companies, and the US government agency [91]. Another multiscale venture is the SMART CAMP (Critical Analytics for Manufacturing Personalized Medicine) involving interdisciplinary research groups (IRG) from MIT and Singapore (Singapore-MIT Alliance for Research and Technology). The SMART CAMP IRG is aimed at developing the technology for the manufacturing of cells for safe, effective, cost-efficient, and personalized medicine.

Future outlook

Microgravity bioreactor

With the advent of manned space flight, it was observed that the astronauts returning from space flight presented with several conditions such as musculoskeletal wastage, anemia, poor immune responses, retinal damage, cardiac insufficiency, and deregulated circadian rhythm [92]. These effects were attributed to the microgravity conditions in space flight (10^{-6} g in space compared to 1 g on earth) and the loss of the radiation shield presented by the atmosphere and the magnetic field of the earth. Several studies were initiated to understand the effects of microgravity on the human physiology, which involved experimentation of human cells in space flight. To circumvent the heavy expenses and long periods of space operations, systems and devices that could generate the microgravity conditions on earth were developed.

The objects aboard the spacecraft in orbital velocity are in a state of free fall; the gravitational force exerted by the earth is continuously counterbalanced by the momentum of the spacecraft [93]. Efforts were focused to facilitate microgravity conditions on earth by employing forces to counterbalance the gravitational force. The first generation of bioreactors SMG by rotating fluid-filled vessels on a horizontal axis, wherein the upward hydrodynamic drag caused by the rotation of the vessel counterbalances the downward forces of gravity [94]. The RWV bioreactor design was improved to achieve efficient and 3D cell interactions, reduce undesired hydrodynamic

shear stresses, and allow spatial colocation of particles of different rates of sedimentation and adequate mass transport and aeration [95]. The rotating wall perfused vessel (RWPV), the slow turning lateral vessel, and the high-aspect rotating vessel (HARV) bioreactors were consequently developed with improvements to the basic design to achieve the aforementioned criteria at microgravity conditions. In ground conditions, at unit gravity (1 g), the convective flow due to the differences in densities of the media and the cells provides sufficient mass transport to sustain cell viability. However, this is negligible in actual microgravity and the mass transport is entirely dependent on the diffusion, which is inadequate to sustain growing masses of cells. Hence for experiments in space or true microgravity, convective flow is intentionally introduced in the RWPV bioreactors to improve the diffusion and mass transport at the cell surface [96].

Toward the late 1980s, it was observed that cells tend to aggregate more when exposed to microgravity [97,98]. With the establishment of the SMG bioreactors, several studies were performed on cells from the cartilage, cardiac, and the gastrointestinal systems, which led to the quick realization that microgravity conditions promoted enhanced cellular interactions and efficient mass transport that led to the formation of cellular aggregates larger than those observed underground conditions. The close positioning of cells and the low shear stress environment likely promotes cell−cell contacts, which mature into cellular junctions that conduct various paracrine signals, which would otherwise be disturbed due to high shear forces operative in conventional bioreactors. These studies were able to identify and address several molecular phenomena influenced by microgravity involved in the differentiation of stem/progenitor cells and tissue development [99−101], metabolism [102−104], immune responses [105,106], wound healing responses [107,108], host−pathogen interactions [109], and cancer biology [110−112].

The effects of microgravity on bone were investigated in space, and it was observed that osteoblasts cells exhibited abnormal shape, reduced stress fibers, poor glucose utilization, and reduced prostaglandin synthesis in microgravity, which explained the osteoporosis observed in astronauts [113]. The HARV bioreactors were employed to understand the effects of hydrodynamic stresses on the proliferation, differentiation, and maturation of the bone marrow stem cells into osteogenic lineages. The bone grafts developed under hydrodynamic conditions repaired bone defects effectively in rat models with better osseointegration compared to grafts obtained from static growth [114]. This study elucidates the potential use of RWV bioreactors for bone tissue engineering. DiStefano et al. (2018) have optimized the rotary conditions for the differentiation of retinal organoids from mouse pluripotent stem cells. The study observed enhanced proliferation and the formation of larger organoids from the stem cells. Also, the differentiation of the retinal organoids in the RWV bioreactors mimicked the spatiotemporal progression of in vivo retinogenesis [115]. Chang and Hughes-Fulford [116] have observed that primary mouse hepatocytes formed 3D aggregates when cultured in RWV, as early as 24 hours compared to growth in conventional tissue culture dishes. The cells in the aggregates attained high functionality in terms of albumin secretion and CYP1A1 enzymatic activity. Interestingly, these effects were independent of cell proliferation and presumably mediated through enhanced cellular interactions. The hepatocytes in the RWV cultures exhibited a unique gene expression signature compared to developing or regenerating hepatocytes [116]. This study clearly implicates that 3D cellular contacts are critical to the functionality of the tissues, which could be induced and maintained by culturing cells in low-shear modulated microgravity bioreactors. Apart from the specific examples discussed above, several other tissue-engineering ventures have been pursued by employing the advantages of the microgravity bioreactors [117,118].

Taken together, RWVs create microgravity by the precise counterbalance of gravitational forces by the angular momentum due to the rotation of the vessels. This leads to quasistationary movement of particles with 3D spatial freedom, low hydrodynamic shear stress, colocation of particles, and efficient mass transport. Modulation of speed of rotation, perfusion mechanisms, and other working parameters allow efficient control over the aforementioned features to fit the cellular systems of interest and for explorations under true microgravity. These advantages allow the employment of actual and SMG bioreactors in investigating molecular mechanisms in various pathophysiological conditions, generate disease models, and in development of functional grafts for tissue engineering.

Real-time assessment in the bioreactor

Preclinical animal models are required for pharmaceutical testing, implant testing, and to understand human physiology and disease biology. However, animal maintenance is time consuming, labor intensive, and expensive [5]. With the guiding principles to Replace, Reduce, and Refine (3Rs) [119], bioreactors are seen as effective in vitro platforms for drug/implant testing with a hope to replace or reduce the use of animal models in the future. Over the past decade, significant improvements in design and construction of bioreactors have been made. Numerous design considerations to the mass transport, physiological cues, and cell environment have been made over the years; however, the ability to monitor the cellular growth, development, and differentiation of the engineered tissue

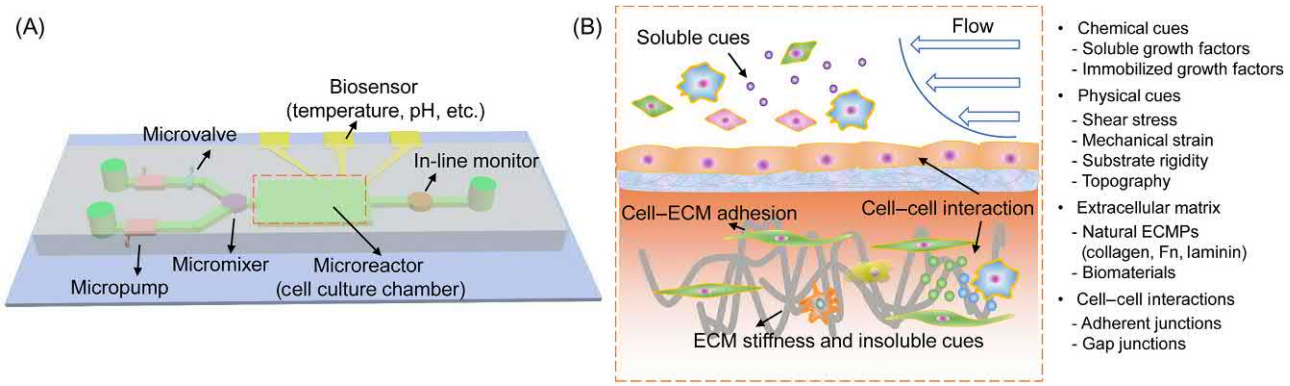


FIGURE 11.4 Some crucial design principles for the microreactors. (A) Different components of a microreactor include microreactor chamber; some fluidic control elements such as micropump, microvalve, and micromixer; biosensors; and in-line monitor element. (B) A detailed illustration of the influential factors for microreactor chamber.

in real time remains a challenge. Traditional methods for evaluating developing tissue in vitro are destructive, time consuming, and expensive [120]. As such, nondestructive, real-time evaluation of the engineered tissue is much desired. In addition, such a facility to image the grafts in the bioreactor environment would add value to the system in terms of evaluating real-time tissue growth and dynamics.

Photoacoustic imaging (PAI) is a laser-generated ultrasound-based technique that is particularly well suited to visualizing microvasculature due to the high optical absorption of hemoglobin [121]. PAI is a nondestructive imaging modality [120], providing the ability to monitor the formation of new blood vessels (angiogenesis) in the tissue engineered scaffold.

Magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), and magnetic resonance elastography (MRE) are noninvasive methods that can provide biochemical, anatomical, and biomechanical measures of tissue [122]. MRS can identify the changing molecular structure and alternations in the conformation of major macromolecules (collagen and proteoglycans) using parameters such as chemical shift, relaxation rates, and magnetic spin couplings. MRI provides high-resolution images whose contrast reflects developing tissue microstructure. These are well-established techniques that have been used to probe the structure and dynamics of biochemical changes in engineered tissues [123–125]. To fully maximize such techniques the engineered tissues should be grown in MR-compatible bioreactors. This will allow for seamless transfer of the samples in the bioreactor chamber for characterization using MRS, MRI, and MRE at the chosen time points and, thereafter, returning the chamber back to the bioreactor to continue cell growth and development.

Lastly, good manufacturing practice (GMP) should also be taken into consideration when designing the bioreactors for such imaging modalities. GMP adheres with stringent guidelines throughout the development and production phases with the intent to achieve quality and safety in the final cell-cultured product. The clinical implementation of bioreactors is expected to be faced with challenges to a certain extent, also due to the logistical set-up and the need for a strong team of well-trained and dedicated cGMP-certified personnel [5]. Whilst the more sophisticated the bioreactor approach, the more likely it is to reflect the natural physiological state, simpler designs are likely to be more operationally robust, so a compromise based on bioreactor complexity versus the essential functional parameters of the desired end-product will always be necessary [126].

Microbioreactors

Design principles

A microreactor for tissue engineering, with dimension smaller than 1 cm, is basically a microdevice in which the organisms are cultivated to recapture the key features of organisms and mimic the real microenvironment for optimal growth and metabolic activity of the organism. These microreactors are beneficial for biophysics researches, underlying the mechanisms of physiological and pathological processes, disease diagnosis and therapy, etc. Many factors need to be considered when designing and fabricating such microreactors, as shown in Fig. 11.4.

Flow rheology

The development of miniaturization technologies had promoted rapid progress in microelectromechanical systems, such as microfluidic chip. Generally, the microfluidic chips consists of microchannels for fluid flow to help mix of liquid and gas, as well as help mass transfer and biochemical reaction. Especially, for microreactors in tissue

engineering, the precise control of the microfluidic flow using micropumps, micromixers, and microvalves require some fundamental understanding of the flow rheology.

The main fluids in the human body that often mimicked in microreactors are blood, urine, tears, perspiration, synovial fluid in the joint, etc., among which most are Newtonian fluid while blood and synovial fluid sometimes can be regarded as non-Newtonian. In detail, blood behaves as non-Newtonian under low shear rates while Newtonian fluid under large shear rates. Synovial fluid, with function of joint lubrication mainly exhibits viscoelastic characteristics, which should be considered for tissue therapy for joint injuries [127,128]. In microfluidics, compared with Newtonian fluids whose viscosities is constant regardless of the shear rate, the rheological properties of non-Newtonian fluids which has nonlinear relationship between the fluid shear rate and its viscosity, are much more complex depending on the chemical compositions, mixture combinations, and many physical conditions such as flow rate and temperature [129]. Normally, the preprocessing (e.g., dilution, washing, enrichment) of the bioassay samples might potentially make a difference in the sample rheology parameters compared to their in vivo status. For example, cell samples are commonly put in buffers such as PBS or DMEM, which are of Newtonian fluid properties. Thus to sufficiently mimic an in vivo environment, rheology must be taken into consideration when designing the microreactors.

Laminar flow or turbulent flow is another factor of flow rheology need consideration for designing bioreactors, which are generally defined by the Reynolds number. Actually, the complex physiologic fluid flow in vivo usually travel through 3D deformable channels, in which distributed turbulent flow might mix with the stable laminar regions. An example is about the pulsatile blood flow, which generally can be regarded as laminar flow in a healthy circulatory system. However, during fraction of each cardiac cycle, small turbulence in the aorta is detected, which will be promoted by the occlusion or stenosis like the stenosis of a heart valve in the circulatory system. Besides, heavy breathing and coughing can also result in turbulent flow [130].

A microreactor system usually consists of some components, such as micromixer, micropump, and microvalve, which can control the flow in order to better control the bioreaction process. The microreactors have some unique advantages, for example, it offers a suitable platform for complex reactions with great performance on controlling the kinetics by improving the bioreaction conditions [131]. Different micromixers have been designed to improve the mixing efficiency including active and passive mixers based on whether need mechanical agitation or external forces. The active micromixers are designed utilizing perturbation force based on electrokinetic, dielectrophoretic, magnetic, and acoustic energy, which usually require special fabrication procedure [132,133]. Passive micromixers produce a laminate of multiple fluid flows, enlarging the interfacial region for fluid diffusion, which is effective for mixing but may cause high pressure drops [133,134]. In addition, the flow in microreactors is categorized into segmented flow and continuous flow, of which the segmented flow includes gas–liquid segmented flow and liquid–liquid segmented flow [135]. For gas–liquid segmented flow, flow modes depend on the surface velocities of gas and liquid. When gas bubble is between two liquid parts, the drag force at the walls enhances convective mixing with each part, narrowing residency time and promoting chemical homogeneity. In liquid–liquid flow, a large specific interface region for immiscible systems generates a short molecular diffusion distance and hinder the unwanted side reactions. While maintaining a continuous stream of the other, multiple pulses of one liquid allow diffusion and reaction to occur at multiple transverse interfaces [133,136,137].

Besides, micropumps and microvalves are also two important elements in microreactors. Micropumps performs well in generating temporal and volumetric fluid movement and on-chip integration can reduce the external hardware for operation, which can be classified as nonpowered and powered ones. In detail the nonpowered pumps (based on osmotic, diffusion, and response to environmental stimuli) do not require electrical actuation and therefore are usually in simple format, easy to fabricate, and possible for minimization. The flow rate mainly determined by the materials, fabrication methods, and is dependent on the fluid properties. Generally, the performance of these micropumps is limited by slow release rates and response [138]. Powered pumps (e.g., electromagnetic, electrostatic, piezoelectric, thermopneumatic, shape memory alloy) often actuated by electricity, heat, or liquid pressure [138,139], which usually have fast response time and large actuation force, with limitations of complex fabrication processes and high driving voltages [139]. Similarly, microvalves control timing, routing, and separation of fluids within a microfluidic device, which can also be categorized as active microvalves and passive microvalves. The active microvalves are divided into nonmechanical (based on hydrogel, paraffin, sol–gel, and ice), mechanical (based on shape memory alloy and thermopneumatic), and external microvalves (e.g., thin membrane or in-line microvalves) [140,141]. On the other hand, the passive microvalves also include mechanical and nonmechanical microvalves. The passive mechanical microvalves mainly work in line with polymerized gel or passive plug, while passive nonmechanical microvalves utilize the surface properties in the microchannels such as hydrophobicity [142,143].

Importantly, the performance of processes in microreactors can be analyzed by a wide variety of simulation software, which can save time and money when designing and fabricating a microreactor with some specific characteristics. In this process, some physical parameters should be early defined such as mass, heat, momentum transfer, kinetic equations as well as boundary conditions [144]. Based on the simulation and computation results, the designing can be optimized accordingly.

Cell microenvironment

Cells are the basic unit of living organisms, residing in a complex microenvironment maintaining cell viability and function and supporting 3D tissue development, where cells encounter complex cues from ECM, fluid stress as well as neighbor cells. These cues include chemical, mechanical, and topographical cues, which are the main determinants of cell phenotype [145,146].

In the cell microenvironment, soluble nutrients and oxygen are crucial factors for tissues culture in vitro. Research showed that there generally was a hypoxic, necrotic center surrounded by viable cells in cellular spheroids if diameter larger than 1 mm [147]. And incorporation of flow in microreactors promotes high mass transfer, which can regulate oxygen concentration, provide nutrients to promote 2D or 3D structure, and remove metabolic products [17]. It's clear that perfusion in microreactors is essential to maintain cell viability and function. However, shear stress is an important factor need to be considered as experimental studies on suspension-adapted mammalian cells found turbulent shear can cause more damages to cells than laminar shear of the same magnitude [148]. Results from studies of shear impact on adherent mammalian cells in 2D and 3D culture demonstrated multiple influences on cell attachment, cell proliferation, and cell differentiation [149–151].

The ECM consists of many proteins and polysaccharides, of which the local extracellular biochemical cues has shown to be an essential factor for tissue development and function, such as TGF-β for mammary branching morphogenesis [152], VEGF for angiogenesis [153], and Wnt proteins for embryo development [154]. Besides, tissues which are located distantly from each other can also have effective communication through circulating soluble factors. In addition, the spatially distributed ECM biochemical cues in vivo are also crucial factors for regulating dynamic cell behaviors, such as cell polarization and migration [155,156]. Many techniques have been developed to create micropatterns of ECM biochemical cues to help study how ECM biochemical cues effect cellular behaviors by dynamic cell–ECM and cell–cell interactions. It helps reveal the function of ECM biochemical properties on physiological and pathological processes, such as wound healing, tissue morphogenesis, and cancer metastasis [157].

Mechanical cues are another important factor for microreactors, which include the mechanical stiffness of ECM and cells or tissues, and the mechanical forces exerted. The ECM and cells or tissues stiffness is their intrinsic mechanical property, for example, mechanical stiffness of mammalian cells in vivo have a wide range from 0.1 to 50 kPa [85,86]. However, the mechanical properties of tissues under pathological condition can significantly alter. For instance, it's reported that compared with normal cells, the cancer cells in tumor stroma are about 5–20 times stiffer compared with their normal counterpart [158]. Besides, research show cancer progression is related with complex interactions between cancer cells and biochemical and biophysical cues of the ECM [159,160]. Importantly, mechanical force is recognized as an important regulator of cell physiological status and promotes the cell activities in bioartificial matrices, further helping in improvement of tissue regeneration in vitro [45]. Mechanical forces in microreactors can be generated from the flow in circulation and compression or stretching of materials contacted with cells. These aspects are essential for consideration when mimicking organ-specific biomechanical cues for microreactors [146,161].

Integration of multiple compartments

Cells in vivo reside in 3D complex microenvironment, including biochemical factors, external mechanical forces, ECM topography, and cell–cell interactions. In microreactor system the integration of multiple compartments helps mimic multiparameter extracellular environment to study the physiological and pathological process.

Recently, with advances in micro/nanofabrication techniques and biomaterials, integrated on-chip cell culture platforms have successfully reconstitute organ-level functions of lungs [162,163], kidneys [164,165], intestines [166], and other organ-on-a-chip models which have mimicked organ-level functions of bile canaliculi [167], cardiac muscle [168], vascular vessels [169], and neural network [170]. The integrative and multiparameter characteristics of in vivo cell microenvironment need consider the critical factors which are necessary for rebuilding specific tissue functions.

Single organ-on-chip systems exhibited importance of cell–cell and cell–ECM interactions in 3D environments. These systems can hardly mimic the interaction between different organs separated physically, but their communication through the circulation system is essential for their specific function. To mimic communications between different organs, Shuler et al. developed a microchip integrated different microtissue components to establish biochemical coupling [171,172], of which the hepatic

module was incorporated to study the metabolism related cytotoxicity of anticancer drugs [171]. Zhang et al. utilized a multichannel 3D cell culture microfluidic system to mimic multiple organs in the human body for potential applications in drug screening, where TGF-β1 was specifically controlled-released inside the lung compartment by gelatin microspheres mixed with cells [173].

Another example is the barrier function, which exists as a significant hallmark for epithelia layer and endothelia layer exhibiting functional and structural polarity. To duplicate the polarized cellular features a general method is using a triple layers structure with a semipermeable membrane located between the two microfluidic channels [174,175]. Based on this, when cells form a coherent monolayer on the semipermeable membrane, both biophysical and biochemical microenvironment cues such as flow rate, osmotic pressure, and cytokine concentrations could be changed independently in the two separate microfluidic channels on top and in the bottom [146].

In addition, many tissues in vivo are more or less affected by mechanical stretches, such as stretching of alveolar cells when breathing and stretching of vascular cells by blood pressure. Reconstructing physiological mechanical stretching is considered especially necessary for replicating functions of these tissues. Cell in situ stretching has already been realized in microreactors via nanomembrane deflection [176] or in-plane membrane stretching [174,177].

Many biomimicry organ-on-a-chip models are ultimately developed for translational researches such as drug screening. For example, Huh et al. developed the lung chip, which had been applied to research on pulmonary edema for developing potential new therapeutics [175]. Toh et al. developed a liver chip based on multiplexed microfluidic channels to maintain the cell synthetic and metabolic functions, aiming for in vitro drug toxicity to predict in vivo drug hepatotoxicity [178]. However, biomimicry organ-on-a-chip research is still immature, which can hardly mimic both organ structures and functions. Considering the current progresses in this field and the development of microengineered functional biomaterials, organ-on-chip platforms recapturing multiparameter microenvironments through integrating micro/nanoengineered biomaterials will benefit fundamental mechanobiology studies as well as translational medicine.

Types of microreactors

The microreactors commonly indicate the bioreactors with dimension smaller than 1 mm, with flow as a significant element, which can be divided into perfusion-based and diffusion-based ones.

For the perfusion chips, the simplest microfluidic configuration is using an internally connected microchannel network via assembly of capillary tubes or an on-chip microchannel network. Generally, chemicals (medium, nutrients, stimuli, etc.) are flowed into a microreactor through tubes connected with syringe pumps, and cell metabolism products and waste medium are collected in the outlets of microreactors. The perfusion-based microreactors show great efficiency in bioreactions, which provide high mass transfer and help cell communication via flow. However, some rheology problems need to be solved before designing and using a perfusion microreactor system, such as laminar flow and reducing the shear stress. With the advances in functional biomaterial, 3D cell culture can be integrated with such perfusion-based microreactors. For example, Domansky et al. and Powers et al. developed perfusion microreactors for studying morphogenesis of 3D tissue structures to direct the hepatic lobule. The morphogenesis of cells is regulated partly by the channel geometry and the chemicals on scaffold surface, which determines the relative values of cell−cell and cell−substrate adhesion force [179,180]. Generally, an optimized perfusion bioreactor for tissue engineering should balance the mass transfer of nutrients and the fluid shear stress within the scaffold pores.

With development of 3D ECM and hydrogel fabrication technologies with microfluidics, 3D cellular microenvironments can also be realized in diffusion-based platforms since most of the proteins and nutrients essential for cell survival are easily diffused in hydrogel [181]. The approach of hydrogel fabrication with microfluidics provides a higher efficient transport for medium and soluble factors, improving control of soluble factor gradients in 2D/3D cell culture, and enabling perfusion of an engineered tissue scaffold [182].

Components and integration into microreactors

In microreactors, fluid is a significant factor both for mass transfer and cell viability. A balance between mass transfer of nutrients and the fluid induced shear stresses is one of the essential factors for designing the microreactors. Some components, such as micromixer, micropump, and microvalve, were integrated with the microreactors for the purpose of fluidic control, which had been introduced previously. In addition to fluidic concentration the concentration distribution is also important factor in microreactors. Based on microfluidic method, stable chemical gradients can be feasibly generated in microreactors. For example, Torisawa et al. fabricated a microfluidic device to study chemotaxis of cancer cells, in which chemical gradient is determined by source and sink microfluidic channels [183]. Allen et al. used a microfluidic perfusion microreactor to generate oxygen gradients to study the regional variations in oxygenation on liver zonation [184].

Cell chambers is another essential component in microreactors, for which some general characteristics are necessary, such as sterilization and nontoxicity of the components, easy assembly and feasibility of parameter monitoring along the medium flow. In the past years, most microsystems for cell culture were designed for 2D monolayer cell culture, with the cell chambers mainly fabricated of PDMS, PMMA, and glass. Recently, biologically derived materials such as collagen gels and matrigel, and synthetic biomaterials such as hydrogels, have been developed for 3D culture in microreactors. Especially, the synthetic biomaterials provide a more controllable physical and chemical microenvironment as multiple parameters could be tuned such as porosity, crosslinking density, and the degradation rate [185,186]. Importantly, 3D ECM and hydrogel fabrication technologies can be integrated into in flow-competent platforms to construct 3D cellular microenvironments [181,187]. For example, a 3D hydrogel that enables spatial control defined by the chambers structure was used directly within cell culture chambers on chip [187]. To fabricate gels with the channels open during the casting process, a pneumatic actuation strategy was used. In another study, microfluidic channels were integrated with a hydrogel and ECM scaffold to mimic a vascular structure [188].

Due to the fact that tissue or cell culture is a dynamic process which could be effected by many parameters, such as temperature, pH value, humidity, nutrient concentration, oxygen concentration, biochemical, and mechanical stimuli. It is expected to endow the microreactors with custom on-line sensors to dynamically monitor and control these parameters. Except for the purpose of monitoring the microenvironment, microsensors can also be applicable for detect and monitor the cell physiological activities and behaviors, such as migration, immune activation, and metabolism. For example, Bavli et al. incorporate liver-on-a-chip system with microsensors to observe changes in glucose and lactate in order to real-time track the dynamics of metabolic adaptation to mitochondrial dysfunction, which allows detecting the chemical toxicity before any observed effects on cell or tissue viability [189]. Lind et al. focused on printing a readable heart-on-a-chip platform with embedded sensors for measuring the contractile force from the laminar cardiac tissue [190]. Zhang et al. developed an integrated modular physical, biochemical, and optical microsensors through a fluidics-routing breadboard, which can operate organ-on-a-chip units in a dynamic and automated manner [191].

Applications

While tissue engineering is traditionally associated with applications in regenerative medicine, it has more recently been explored as a method to develop physiological models of disease. These models have been proposed to have utility for a wide range of functions, including drug testing and screening, experimental models to study disease biology, as well as clinical prognostics and diagnostics for personalized medicine. Microreactors often take the form of "organ-on-chips" and a summary of the strengths and limitations of these systems are presented in Table 11.3.

Drug testing and screening

Newly developed drugs require extensive tests and evaluations before they can be allowed to enter the market. These activities typically include preclinical trials involving in vitro and animal models to study the biochemical and physiological effects; in order to proceed to trials in humans, the ensuing clinical trials often take many years and are extremely expensive. However, more than 90% of the new drugs are failed at this stage as they are unable to demonstrate real health benefits, suggesting the inadequacy of existing preclinical models to accurately represent the true safety and efficacy in humans. Reasons for this include the inability of traditional 2D cell cultures (most often used in early tests of compound efficacy and

TABLE 11.3 Summary of the strengths and limitations of organ-on-chips systems.

Strengths	Limitations
Provide perfusion flow to maintain three-dimensional tissue structures and simulate blood flow	Technically challenging to scale
Complexity allows greater approximation to physiological systems for modeling of ADME	Reproducibility compromised by complexity of system
Controllable flow conditions to match physiological parameters	Long-term cultures remain vulnerable to contamination
Accessibility of on-chip tissue for repeated sampling	Laborious procedures for preparation and maintenance of system

ADME, Absorption, distribution, metabolism, and excretion.

toxicity) to recapitulate the spatial complexity seen in vivo. In contrast, animal models often do not capture human-specific drug responses or specific features of a target disease. It follows that more stringent evaluation of drug responses may prove valuable as an approach to "fail" candidate compounds at an earlier stage, avoiding the heavy costs associated with attrition in the latter stages of drug development.

To this end, organs-on-chips models, in which tissue-specific cells are cultured within microfluidic devices, have been developed to provide biomimetic physiological analogs amenable to pharmacological modulation and monitoring of responses. Such models may have important applications in predicting tissue toxicity. To meet the need for assays of chronic hepatotoxicity, for example, advanced cell culture techniques have been developed that integrates incubator features into microfluidic devices, facilitating extended, continuous culture periods for maintenance of 3D hepatocyte cultures [192]. Following optimization of flow conditions to remove metabolic by-products and reduce shear stress on cultured spheroids, cellular viability, and functionality were demonstrated for on-chip hepatocyte spheroids after more than 2 weeks in culture. The resulting devices were used for acute and chronic repeat dosing drug safety testing of diclofenac and acetaminophen. Crucially, diclofenac toxicity is idiosyncratic and cannot be reproduced reliably in animal models; using the perfusion incubator liver chip model, the authors found a more sensitive chronic toxicity response compared to static controls.

Microfluidic devices containing multiple, connected organ compartments have been developed in the more ambitious "body-on-chip" models, which may be used to observe ADME (absorption, distribution, metabolism, and excretion) pharmacology. Four-organ chips have been developed comprising human intestine, liver, skin, and kidney equivalents, in which drugs could be delivered via a reservoir at the apical surface of the engineered intestinal wall to simulate oral administration [193]. The "absorbed" drug is sequentially delivered to liver, skin, and kidney equivalents, representing primary metabolism, secondary metabolism, and excretion, respectively. The amenability of the different compartments to repeated sampling provides the unique opportunity to evaluate pharmacokinetic and pharmacodynamics parameters in real time.

Experimental models of disease

Animal disease models are used in a variety of settings in basic research, such as studies on disease etiology and mechanisms of disease progression. Limitations of using animals for research include high costs, ethical concerns over the use of research animals, inability to recapitulate disease states faithfully, and limited tools for in vivo experimentation. Taking early events in bone metastasis as an example, animal models lack fidelity in reproducing bone metastasis events and are generally inaccessible to real-time, single-cell imaging [194].

The first organ-on-chip device was a lung model where the authors were able to demonstrate reproduction of a mechanically active alveolo-capillary membrane, the smallest functional unit in the lung [174]. Using this model, the team was able to demonstrate the nanotoxicity of inhaled silica nanoparticles. In addition, because the mechanical forces were externally controlled, the effect of mechanical strain on nanoparticulate uptake could be decoupled and independently assessed, leading to the finding that cyclic stretch increased nanotoxicity and associated inflammatory responses.

Pathological tissue may also be directly cultured on chip, as seen with models focused on representing specific steps in the cancer cascade. Cancer-on-chip models have been developed to study tumor growth, progression through epithelial-mesenchymal transition, intravasation into vasculature, and metastasis. In particular, cellular dormancy is a feature of disseminated tumor cells in the premetastatic niche and is believed to be influenced by proximity to perivascular niches and is often studied using 2D cell cultures [195]. To recapitulate this in lung-on-chip systems, lung cancer cells can be orthotopically introduced into the primary lung alveolar or small airway epithelial cells, which integrate into the tissue layers. Using this model, it was found that cancer cell growth was accelerated in the lung alveolus microenvironment compared to in the lung airway, where the inoculated tumor cells remained largely dormant [196]. Corollary to advancements in the development of multicompartmental body-on-chip systems, orthotopic primary tumor models may be generated in one organ chip and subsequently followed through the metastatic cascade. Such models will have critical utility in understanding cellular and molecular mechanisms and interactions across the various stages of cancer metastasis.

Prognostic/diagnostic tools

As a medical model, precision medicine (PM) proposes customized health care according to the individual patient. The motivation for PM arises from the recognition of a significant proportion of nonresponders within a patient population and the need to reduce prescription of treatment regimes that are ineffective or harmful. Indeed, an analysis of bone biopsies obtained from 189 patients with advanced, bone-metastatic prostate cancer showed that up to 89% of affected individuals had a "clinically actionable outcome" [197]. Thus genomic profiling, biomarkers, and biometrics may be useful in stratifying patients according

to the likelihood of response, in order to achieve personalization of their treatment regimes. These data, however, often only show a snapshot of a static time point and offer little in representing the complexity of the disease.

Functional testing, in which patient-derived tissues are used for evaluation of functional response to novel drug treatments, addresses this by recapitulating some of this complexity, such as heterocellular interactions. To generate personalized organs-on-chips for patients, primary tissue may be obtained and cultured on microfluidic systems. However, cells derived from primary sources have limited capabilities to grow and an alternative approach to transform patient-derived cells into iPS cells has yielded an alternative. These stem cells may also be further augmented through genetic modifications including reporter genes, raising the possibility of real-time monitoring. This technology and approach is still new however, and significant research and development must be undertaken to overcome existing limitations.

Summary

This chapter has explored the principles in developing bioreactors for tissue-engineering applications to mimic the native tissue environment and physiological functions. The bioreactor technologies include macrobioreactors that are primarily utilized to grow functional tissues for in vivo implantation, as well as microbioreactors, which is used to create native tissue-like environment for drug testing and other in vitro studies on biochemical and mechanical regulation of cell responses and tissue development. Clinicians, engineers, and scientists are aiming to continuously advance the bioreactor systems toward (1) developing a robust platform for generation of functional tissue grafts for clinical application, (2) studying diverse biological responses in tissue development and regeneration, (3) optimizing cell manufacturing processes for large-scale, standardized, and reproducible production of high-quality and clinically relevant cells for regenerative medicine. The advancements in specialized bioreactor designs are evident with the growing research on space bioreactors to simulate microgravity on earth so as to study the physiological responses of cells and tissues under such conditions, as well as the advent of integrating the real-time imaging in the bioreactors to monitor the graft development and the tissue growth and dynamics. Developments in the multiple organ-on-a-chip platform gives promise toward developing body-on-a-chip platforms for high throughput drug testing and screening. The nascent developments in the personalized patient-derived organ-on-a-chip platforms as prognostic or diagnostic tool would help to progress the future of bespoke medicine.

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Chapter 12

Regulation of cell behavior by extracellular proteins

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Introduction

The extracellular milieu is a critical determinant of cell behavior in all tissues. Many factors comprise the environment and each factor contributes unique influences on cell behavior. For example, cell:cell contact, cell:extracellular matrix (ECM) interaction, and ECM composition influence cell activity. Environmental components act together to regulate cell surface protein activity, intracellular signal transduction, and subsequent gene expression, which leads to proliferation, migration, differentiation, and ultimately the formation of complex tissues. Matricellular proteins are secreted, modular proteins that are associated with the ECM, are known to influence cell adhesion, cell:ECM binding, and ECM structure and function [1]. This chapter will focus on matricellular proteins as modulators of extracellular signals. A unifying theme of matricellular proteins is to provide a link between the ECM and cell surface receptors, and/or cytokines and proteases localized in the extracellular environment, the activity of which might be affected by this interaction. Thrombospondins-1 and -2, tenascin-C, osteopontin, and secreted protein acidic and rich in cysteine (SPARC) are representatives of this class of proteins. A significant body of evidence points to these proteins as critical mediators of cell signaling pathways and ECM assembly and turnover. Consequently, strategies designed to recapitulate tissue conditions should consider the influence and addition of matricellular proteins to aid in the acclimation of cell populations to engineered environments. In addition, the widespread increases in expression of matricellular proteins in adult tissues undergoing ECM remodeling events, such as those in tissue repair, cancer progression, and fibrosis, identify matricellular proteins as recognized markers of disease onset and, in some cases, resolution (Table 12.1).

Thrombospondin-1

Thrombospondin-1 is a 450,000 Da glycoprotein with seven modular domains [2]. To date, five different paralogs of thrombospondin have been identified, termed thrombospondins-1−5. This chapter will review the two more characterized forms, thrombospondins-1 and -2. At least five different ECM-associated proteins are able to bind to thrombospondin-1: collagens I and V, fibronectin, laminin, fibrinogen, and SPARC [2]. Likewise, cell surface receptors for thrombospondin are multiple and include cluster designation (CD)36, CD47, and the integrin family of ECM receptors [3]. Given the significant number and variety of thrombospondin-1-binding proteins, it is of little surprise that a wide variety of functions have been attributed to this protein, some of which appear to be contradictory. Many of these disparities may, however, reflect dynamic interactions of thrombospondin-1, or cleaved fragments of thrombospondin-1, with other extracellular factors, which can influence cells in different ways to give rise to distinct cellular outcomes—a common theme in matricellular protein biology.

The antiangiogenic properties of thrombospondin-1 are well characterized [3]. In fact, thromospondin-1 was the first identified naturally occurring inhibitor of angiogenesis. In vitro, aortic endothelial cells transfected with antisense thrombospondin-1 cDNA generate twice as many capillary-like structures on a gelled basement membrane in comparison to control cells that produce higher levels of thrombospondin-1 [4]. In accordance, tumors grown in thrombospondin-1-null mice display enhanced angiogenesis accompanied by increases in tumor size. Increased expression of thrombospondin-1 by tumor cells results in decreased vascularization and an accompanied reduction in tumor progression by a number of different

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TABLE 12.1 Matricellular protein interactions and activities.

	Extracellular matrix interaction	Receptor	Adhesive (+) versus counter-adhesive (–)	Growth factor modulation	Extracellular matrix formation
Thrombospondin-1	Col I and V Fn, Ln, Fg	Integrin CD36 CD47 LRP	(–)	HGF (–) TGF-β (+)	+
Thrombospondin-2	MMP-2	LRP CD36	(–)	?	+
Tenascin-C	Fn SMOC1 Perlecan	Integrin Annexin II	(–) (+)	EGF (+) bFGF (+) PDGF (+)	+
Osteopontin	Fn, Col I, II, III, IV, and V	Integrin CD44	(+)	?	+
SPARC	Col I, III, IV, and V	Stabilin-1 VCAM	(–)	bFGF (–) VEGF (–) PDGF (–) TGF-β (+)	+

A summary of the activities for the matricellular proteins described in this chapter. This table is not a complete list of all activities and receptors for the proteins nor all the matricellular proteins included. For references refer to the text. *bFGF*, Basic fibroblast growth factor; *CD*, cluster designation; *Col*, collagen; *EGF*, epidermal growth factor; *Fg*, fibrinogen; *Fn*, fibronectin; *HGF*, hepatocyte growth factor; *Ln*, laminin; *LRP*, lipoprotein receptor-related protein; *MMP*, matrix metalloproteinase; *PDGF*, platelet-derived growth factor; *SMOC*, SPARC related modular calcium-binding protein; *SPARC*, secreted protein acidic and rich in cysteine; *TGF*, transforming growth factor; *VCAM*, vascular cell adhesion molecule; *VEGF*, vascular endothelial growth factor. Growth factor abbreviations are defined in the text.

types of tumor-derived cells representing the potential translational applications for designing antitumor therapies with cell-binding sequences derived from thrombspondin-1 [5].

One mechanism by which thrombospondin-1 might influence angiogenesis is by promoting cell death in microvascular endothelial cells [6]. CD36, a thrombospondin-1 cell surface receptor, has been shown to be required for caspase-3-mediated induction of apoptosis by thrombopspondin-1. Antimigratory effects elicited by CD36 interaction with thrombospondin-1 in endothelial cells has also been demonstrated. A complex containing CD36 and β1 integrins on cell surfaces can collaborate in thrombospondin-1 (and -2) cell signaling [7]. In addition, interactions with the CD36/integrin complex affect vascular endothelial growth factor (VEGF) activity. In fact the balance between TSP-1 and VEGF in the extracellular space is proposed as an important cellular mechanism in angiogenic control [8].

Another thrombospondin receptor, CD47, has been implicated in cardiovascular function as a potent regulator of nitric oxide [9]. Engagement of CD47 by thrombospondin-1 inhibits NO production. As NO is known to be significantly decreased in cardiovascular tissues in aging, the age-dependent increase in thrombospondin-1 has been implicated as mechanism that might account for deficient NO signaling in chronic diseases of aging. As the first natural inhibitor of angiogenesis, characterization of the mechanisms by which thrombospondin-1 functions provides valuable insight into strategies to control blood vessel formation.

Thrombospondin-1, like many of the matricellular proteins, influences cytokine activity. For example, thrombospondin-1 has been shown to interact specifically with transforming growth factor (TGF)-β [10]. This interaction leads to activation of the latent form of TGF-β, presumably through a conformational change in the cytokine, which allows interaction with cell surface receptors [10]. Consequently, the presence of thrombospondin-1 in the extracellular milieu might affect the activity of this potent, multifunctional cytokine. Generation of a thrombospondin-1-null mouse lends support to the importance of thrombospondin-1-mediated activation of TGF-β in vivo. The phenotype manifest in the absence of thrombospondin-1 expression mimics to some degree the phenotype of the TGF-β − null mouse [11]. Specifically, similar pathologies of the lung and pancreas are observed in both mice and, importantly, thrombospondin-1-null mice treated with thrombospondin-1 peptides show a partial restoration of active TGF-β levels and a reversion of the lung and pancreatic abnormalities toward tissues of wild-type mice. Importantly, thrombospondin-1 is a potent chemoattractant for inflammatory cells. Therefore a reduction in active TGF-β levels in thrombospondin-1-null mice might be influenced by inflammatory cell recruitment.

The function of thrombospondin-1 in the activation of TGF-β appears to be of greater significance in some tissues in response to injury in adulthood rather than in development. For example, in the heart, thrombospondin-1-null mice have an altered response to both pressure overload (PO)—induced hypertrophy and to myocardial infarction in comparison to the response in wild-type animals although uninjured adult null hearts appear grossly normal. In hearts with PO an increase in the number of myofibroblasts is found in thrombopsondin-1 null versus wild type in the absence of increases in fibrillar collagen [12]. As myofibroblasts are frequently associated with sites of robust collagen production, the lack of increased collagen suggests that myofibroblast differentiation is impaired in the absence of thrombopsondin-1. In fact, thrombopsondin-1-null myofibroblasts from PO hearts are smaller and produce less collagen type I than those from wild-type counterparts. TGF-β is known to induce myofibroblast differentiation; hence, the authors of this study conclude that inefficient TGF-β activity in the absence of thrombopsondin-1 likely influences myofibroblast phenotype in PO hearts.

Thrombospondin-1 influences cell adhesion and cell shape. For example, it will diminish the number of focal adhesions of bovine aortic endothelial cells and thus will promote a migratory phenotype [13]. Thrombospondin-1, therefore, is proposed to modulate cell:matrix interaction to allow for cell migration when necessary. An intermediate stage of adhesion describes cell responses to counter-adhesive matricellular proteins and, as such, is predicted to promote cell motility [13]. Accordingly, thrombospondin-1 expression is observed during events such as dermal wound repair, when cell movement is required. Thrombospondin-1 is also expressed by many tumor cells and might facilitate metastatic migration [7].

Thrombospondin-2

Similar to thrombospondin-1, thrombospondin-2 inhibits angiogenesis in vivo. Recombinant thrombospondin-2 contained in pellets implanted in the rat cornea inhibits basic fibroblast growth factor (bFGF)—induced vessel invasion to nearly the same degree as thrombospondin-1 [14]. Thus the observation that the thrombospondin-2-null mouse appears to have a greater degree of vascularization in the skin is in accordance with the hypothesis that thrombospondin-2 might serve as an endogenous regulator of angiogenesis in mice [15]. In fact, the expression pattern of thrombospondin-2 is more consistent with the importance of this protein in vascular formation, as the mRNA for thrombospondin-2 is more closely associated with the vasculature in developing tissues, in comparison to that of thrombospondin-1 [16].

How might thrombospondin-2 mediate inhibition of angiogenesis? Abrogation of thrombospondin-2 expression results in increased amounts of matrix metalloproteinase (MMP)-2 activity [17]. Thrombospondin-2 binds to MMP-2 and facilitates uptake by scavenger receptors on cell surfaces. Hence without thrombospondin-2, MMP-2 levels accumulate in the extracellular milieu and result in significant decreases in cell adhesion. The increase in MMP-2 levels is correlated with decreased amounts of tissue transglutaminase on thrombospondin-2-null cell surfaces [18]. As transglutaminase is known to enhance integrin-mediated cell attachment and serve as a substrate of MMP-2, the increase in MMP-2 activity is proposed to elicit decreased cell adhesion in the absence of thrombospondin-2 by diminishing amounts of transglutaminase on cell surfaces. Hence, blood vessel formation appears to be sensitive to levels of MMP-2 in tissues. Likewise, tissue transglutaminase on cell surfaces might also contribute to angiogenic events.

Another observation from the thrombospondin-2-null mouse is that of altered collagen fibrillogenesis in the skin, relative to that seen in wild-type mice. The collagen fibrils in the null mice are larger in diameter, have aberrant contours, and are disordered [15]. Presumably this effect on collagen fibril formation contributes to less tensile strength of the skin in thrombospondin-2-null mice versus their wild-type counterparts. An intriguing relationship between aberrant collagen fibril morphology and decreased transglutaminase activity is suggested by the thrombospondin-2-null phenotype. Evidence also suggests that thrombospondin-2 is a profibrotic molecule. In the absence of thrombospondin-2, less collagen deposition is found in a murine cardiac graft model [19], whereas increased thrombospondin-2 levels in scleroderma fibroblasts contribute to increased collagen production through upregulation of miR-7 [20]. Evidence that changes in thrombospondin-2-null ECM might reside in differences in miR-29 expression is also reported [21].

In tissue engineering applications, ECM derived from the skin of thrombospondin-2-null mice supports greater cell migration versus that from wild-type mice when implanted subcutaneously in mice [22]. The decellularized dermal matrix is applied as either a slab or as comminuted powder delivered in silicone trays. Importantly, the thrombospondin-2-null matrix showed improves efficacy in healing diabetic wounds in a murine model. As diabetic animals have notably impaired healing ability, improvements brought about with the thrombospondin-2-null ECM has potential translational applications. In addition to the lack of thrombospondin-2, the dermis of thrombospondin-2-null mice also differs from wild-type skin in terms of levels of collagens, collagen cross-linking, proteoglycans, and matricellular proteins [21]. Hence, likely a variety of factors in addition to the

absence of thrombospondin-2 confer the improved wound healing capabilities of the decellularized matrix. These studies also highlight the influence that expression of one matricellular protein, in this case thrombospondin-2, can have on the overall composition of the ECM [21].

Thrombospondins-1 and -2 can act as negative regulators of cell growth. In particular, endothelial cells are susceptible to an inhibition of proliferation by both proteins, resulting in their classification as inhibitors of angiogenesis. However, the variety of cell surface receptors for thrombospondins allows for diverse signaling events in different cell types; consequently, there might be situations when thrombospondin appears to support angiogenesis as well [23]. Thrombospondin-1 has been shown to modulate the activity of at least two cytokines, TGF-β and hepatocyte growth factor (HGF): diminished activity, in the case of HGF, or enhanced activity, as seen for TGF-β. Finally, thrombospondin-1 can not only alter cell shape to promote a migratory phenotype but also inhibit migration. Such conflicting conclusions illustrate the importance of contextual presentation of matricellular proteins in various assays. Further characterization of the thrombospondins, including closer examination of the remaining family members (thrombospondins-3−5), will no doubt yield fascinating insight into this multifunctional gene family [24,25].

Tenascin-C

Tenascin-C is a matricellular protein with a widespread pattern of developmental expression, in comparison to a restricted pattern in adult tissues. In addition to tenascin-C, three other, less-characterized forms of tenascin have been identified: tenascin-R, tenascin-X, and tenascin-W [26]. This section will focus on tenascin-C, as the best characterized of the tenascin gene family. Tenascin-C consists of six subunits (or arms) linked by disulfide bonds to form a 2000 kDa molecule that can associate with fibronectin in the ECM [27]. Like thrombospondin-1, a number of different functions have been attributed to tenascin-C and, accordingly, a number of cell surface receptors appear to mediate distinct properties of this matricellular protein. Cell surface receptors for tenascin-C include annexin II and at least five different integrins receptors, of which $\alpha_9\beta_1$ demonstrates the highest affinity for tenascin-C [28]. While the integrins appear to support cell adhesion to tenascin-C, annexin II is thought to mediate the counter-adhesive function attributed to this protein. Hence, tenascin-C can act as either an adhesive or as a counter-adhesive substrate for different cell types, dependent upon the profile of receptors expressed on the cell surface.

Tenascin-C has also been shown to modulate the activity of growth factors: specifically, it promotes epidermal growth factor (EGF)−dependent and bFGF-dependent cell growth [29,30]. In fact, Jones et al. [30] have shown that smooth muscle cells plated in a collagen gel secrete MMPs that degrade the collagen to expose integrin receptor−binding sites. Engagement of these receptors induces tenascin-C expression; tenascin-C is subsequently deposited into the ECM and can itself serve as an integrin ligand. The deposition of tenascin-C leads to cell shape changes initiated by a redistribution of focal adhesion complexes concomitant with a clustering of EGF receptors on the cell surface. Presumably, clustering of the EGF receptors facilitates EGF signaling and thereby enhances the mitogenic effect of EGF. Conversely, when MMP activity is inhibited, tenascin-C expression is decreased and the cells become apoptotic [30]. Thus tenascin-C is able to modulate EGF activity such that the presence of this matricellular protein supports cell growth and its absence induces programed cell death.

Similarly, tenascin-C supports tumor metastatic colonization by breast cancer cells through suppression of cell apoptosis [31]. Tenascin-C expression by stromal cells as well as by metastasis-initiating breast cancer cells appears to protect tumor-derived cells from apoptotic stresses and thereby facilitates establishment of metastatic colonization in some tissues [32,33]. A function of tenascin-C in hematopoiesis following myeloablation in adult mice is also reported. Tenascin-C expression in the bone marrow niche microenvironment is required for regeneration of hematopoiesis after ablation but is not required to maintain steady-state conditions [34].

Given the widespread expression of tenascin-C in the developing embryo, the lack of an overt phenotype in the tenascin-C-null mouse is surprising [35]. In particular, the high level of tenascin-C expression in the central and peripheral nervous system had indicated that the absence of this protein might lead to neuronal abnormalities. Although no histological differences could be detected in the brains of adult tenascin-C-null mice, they displayed behavioral aberrations, including reduced anxiety and enhanced novelty-induced activity [36,37]. In addition, altered numbers of embryonic central nervous system stem cells are noted in the absence of tenascin-C expression, an observation confirming that the composition of the ECM is an important factor in cell differentiation [38].

The genetic background of the tenascin-C-null mouse is likely to be a major factor in the identification of tissues in which tenascin-C might be functionally important. For example, Nakao et al. [39] use three different congenic mouse lines to study the effect of Habu snake venom−induced glomerulonephritis in a tenascin-C-null background. Although the disease is worse in all tenascin-C-null mice in comparison to wild-type controls, each

line exhibits a different level of severity. Induction of the disease in one strain, GRS/A, results in death from irreversible renal failure [39]. Moreover, mesangial cells cultured from tenascin-C-null animals do not respond to cytokines, such as platelet-derived growth factor (PDGF) unless exogenous tenascin-C is included in the culture medium. Hence, tenascin-C can also modulate the activity of this growth factor as observed previously for EGF. Tenascin-C provides another example of a matricellular protein able to affect growth factor efficacy.

Although tenascin-C shows a limited pattern of expression in the adult, an induction of tenascin-C is seen in many tissues undergoing wound repair or neoplasia [40]. Thus tenascin-C, like the other matricellular proteins, is ideally suited to act as a modulator of cell shape, migration, and growth. Importantly, tenascin-C can be used as a marker of successful tissue repair. One mechanism by which tenascin-C influences cell behavior is through the modulation of fibronectin interaction with cells. Tenascin-C decreases cell adhesion to fibronectin through competition with a heparan sulfate proteoglycan, syndecan 4 [41]. Syndecan 4 is required for efficient cell attachment to fibronectin and for tenascin-C inhibition of adhesion to fibronectin. Hence, a scenario in which tenascin-C competes for syndecan 4 binding to fibronectin is consistent with these results. Certain proteolytic fragments of tenascin-C interfere with fibronectin assembly although full-length tenascin-C does not demonstrate inhibition of fibronectin fibrillogenesis [42]. Given the high levels of proteolytic activity in wounds, for example, cleavage of tenascin-C might be one mechanism by which regulation of fibronectin assembly is achieved. Interestingly, molecular phylogeny indicates that tenascin-C evolved prior to fibronectin; hence, tenascin-C is likely to have functions unrelated to fibronectin at some level.

Nonetheless, tenascin-C production is aligned with improved repair and resolution in many tissues. Fragments of tenascin-C have been exploited in bioengineering applications to enhance cell activity. For example, the integrin activating tenascin-C peptide, when incorporated into bioengineered gels, enhances neurite outgrowth, a quality that is improved in three-dimensional (3D) cultures [43,44].

The paucity of developmental abnormalities manifested in the tenascin-C-null mouse points to the greater importance of tenascin-C in remodeling events, which take place in response to injury or transformation. For example, tenascin-C has been shown to regulate cardiac neovascularization by bone marrow−derived endothelial progenitor cells in response to angiogenic stimuli in adult mice [45]. In addition, as mentioned, high levels of tenascin-C expression are frequently associated with several types of malignancies, including tumors of the brain and breast [26]. In fibrosis, tenascin-C expression is implicated in a positive feedback loop in fibroblasts that involve Twist1 and paired-related homeobox 1 (Prrx1) in which chronic activation of fibroblasts leads to excess collagen deposition [46].

Osteopontin

As the name implies, osteopontin was originally classified as a bone protein. A more thorough examination, however, reveals a widespread expression pattern for this protein with multiple potential functions [47]. Osteopontin associates with the ECM, as it binds to fibronectin and to collagens I, II, III, IV, and V. Osteopontin also affects cellular signaling pathways by virtue of its capacity to act as a ligand for multiple integrin receptors as well as (CD)-44 [47]. Thus osteopontin, like most of the matricellular proteins, is able to act as a bridge between the ECM and the cell surface. Since matricellular proteins might be synthesized, secreted, and incorporated into the ECM with greater ease than more complex structural ECM proteins that must be incorporated into fibrils and assembled into a network, a bridging function might be useful during remodeling events in the organism when rapid conversion of the cellular substrata is required for cell movement.

Osteopontin serves as an integrin ligand through an RGD sequence and a SVVYGLR sequence located near the thrombin cleavage site. Osteopontin is also susceptible to modification by ECM metalloproteases [48]. In support of osteopontin enhancing cell adhesion, Weintraub et al. [49] report that transfection of vascular smooth muscle cells with antiosteopontin cDNA reduces adhesion, spreading, and invasion of 3D collagen matrices. Addition of osteopontin to the collagen gel restores the capacity of these cells to invade the gel [49]. In addition, Senger et al. [50] report that endothelial cells treated with VEGF increase their expression of the integrin $\alpha_v\beta_3$, an osteopontin cell surface receptor, concomitantly with an increase in osteopontin. These investigators also show an increase in the amount of thrombin-processed osteopontin in tissues injected with VEGF and radiolabeled osteopontin. The significance of this result lies in the enhanced support of endothelial cell migration in vitro by thrombin-cleaved versus full-length protein [50].

In pulmonary hypertension, adventitial fibroblasts from pulmonary arteries demonstrate an activated phenotype characterized by high rates of proliferation and migratory potential. High levels of osteopontin expression are correlated with this activated phenotype [51]. Furthermore, silencing of osteopontin expression significantly reduces proliferation and migration in hypertensive fibroblasts, whereas increased osteopontin activity stimulated proliferation and invasion by normal fibroblasts [51]. Thus osteopontin is predicted to be a primary

determinant of the activated phenotype characteristic of adventitial fibroblasts in hypertensive lungs. Increased levels of osteopontin secreted by senescent fibroblasts also promote migration of mammary epithelial cells, a process linked to neoplastic progression and tumor development in aging tissues [52].

The capacity of osteopontin to influence cell migration might be linked to MMP activity. Osteopontin is a member of the SIBLING (small integrin-binding ligand N-linked glycoprotein) family of proteins that have been shown to bind to and regulate the activity of MMPs [53]. Osteopontin binds to recombinant proMMP-3 and active MMP-3. In addition, a decrease in MMP-9 activity has been reported in osteopontin-null myofibroblasts and vascular smooth muscle cells [54]. Hence, osteopontin serves to both influence extracellular protease activity as well as being susceptible to degradation and functional activity by proteases [65].

The promotion of cell survival is another property ascribed to osteopontin. Denhardt and Noda [55] have reported that human umbilical vein endothelial cells plated in the absence of growth factors will undergo apoptosis. If these cells are plated on an osteopontin substrate, however, apoptosis is inhibited [55]. Furthermore, rat aortic endothelial cells subjected to serum withdrawal undergo programed cell death, a response inhibited by an osteopontin substratum. In fact, it is the ligation of integrin $\alpha_v\beta_3$ by osteopontin at the cell surface that induces nuclear factor-kappa B, a transcription factor that controls a variety of genes through direct binding to their promoters. Thus osteopontin and other $\alpha_v\beta_3$ ligands can protect cells from apoptosis [56].

The function of osteopontin to influence inflammatory response is well documented. Expression of osteopontin is found to increase during intradermal macrophage infiltration, and purified osteopontin injected into the rat dermis leads to an increase in the number of macrophages at the site of administration. Importantly, antiosteopontin antibodies inhibit macrophage accumulation in a rat intradermal model after a potent macrophage chemotactic peptide is used to induce an inflammatory response [57]. Osteopontin expression is required for the development of an effective T_H1 immune response [58]. In glioblastoma, integrin $\alpha_v\beta_5$ highly expresses on glioblastoma-infiltrating macrophages. Wei et al. recently report that reduced levels of osteopontin associated with glioblastoma tumors lead to a significant decrease in M2 polarized macrophages in glioma [59].

The phenotype of the osteopontin-null mouse supports the hypothesis that osteopontin affects macrophage activity. Although the number of macrophages does not appear to differ significantly in incisional wounds of wild-type and osteopontin-null mice, the amount of cell debris was higher in wounds of the latter animals. Because macrophages are thought to be primary mediators of wound debridement, osteopontin is predicted to be important in the regulation of macrophage function [60]. Collagen fibril formation in the deeper dermal layers of wounded osteopontin-null mice also appear to be affected. Osteopontin-null mice have smaller collagen fibrils compared to wild-type controls. Similar to thrombospondin-2, osteopontin might affect collagen fibrillogenesis especially at wound sites, as no differences are seen in the size of collagen fibrils in unwounded skin [60].

The proinflammatory and profibrotic properties of osteopontin suggest osteopontin might contribute to human diseases in which inflammation and fibrosis contribute to pathological processes such as in systemic sclerosis (SSc). In fact, high levels of circulating osteopontin are present in patients with SSc. Osteopontin-null mice, when challenged with bleomycin to induce dermal fibrosis, exhibit significantly reduced levels of collagenous ECM and fewer macrophages [61]. Similarly, in a renal model of fibrosis, significantly less interstitial fibrosis and inflammatory infiltration occur in osteopontin-null mice in comparison with wild-type animals [62].

Another primary function of osteopontin is to serve as a negative regulator of calcification [63]. Osteopontin inhibits apatite crystal formation in vitro and facilitates resorption of cellular minerals. As shown in an in vivo model of ectopic calcification, the capacity of osteopontin to abrogate calcification is dependent upon the phosphorylation of osteopontin and the presence of the integrin-binding RGD sequence in osteopontin [64]. In mineralized tissues, osteopontin is an important factor in the regulation of bone turnover whereas in nonmineralized tissues, osteopontin might influence ectopic calcification events [65].

Osteopontin and its repertoire of cell surface receptors represent components of a pathway used by cells in need of rapid movement or migration. In addition, the ligation of osteopontin by certain cell surface receptors supports cell survival and thus provides a mechanism for a given tissue to protect a subset of cells, expressing the appropriate receptor, from apoptosis. Interestingly, an intracellular form of osteopontin has been implicated as the mediator of interferon-α production in plasmacytoid dendritic cells [66]. Recently, distinct roles of intracellular versus secreted osteopontin were uncovered in the regulation of myeloid versus lymphoid immune response to pathological insult [67]. That matricellular proteins might play vital roles inside the cell, which has been proposed for other matricellular family members as well, including recent reports on the thrombospondin family, represents an exciting area of research [25].

Secreted protein acidic and rich in cysteine

SPARC (BM-40; osteonectin) was first identified as a primary component of bone but has since been shown to

have a wider distribution [68]. Increased expression of SPARC is observed during development and in many adult tissues undergoing different types of remodeling. For example, SPARC is found in bone and in gut epithelium, two tissues that normally exhibit rapid turnover, and in healing wounds. An increase in SPARC is observed in the vast majority of fibrotic conditions and in association with many different tumors [69]. Like other matricellular proteins, SPARC interacts with the ECM by binding to collagens I, III, IV, and V [68].

SPARC has also been shown to bind to a variety of growth factors present in the extracellular space [70]. For example, SPARC binds to PDGF-AB and -BB and prevents their interaction with PDGF cell surface receptors. PDGF-stimulated mitogenesis is inhibited by the addition of SPARC [71]. SPARC can also prevent VEGF-induced endothelial cell growth, as it binds directly to the growth factor and thereby prevents VEGF receptor stimulation of the mitogen-activated protein kinases, Erk-1 and Erk-2 [72]. In vivo, regulation of VEGF activity by SPARC via VEGF receptor-1 occurs during choroidal neovascularization after injury [73].

Interestingly, a variety of mitogenic stimulators are inhibited by SPARC in culture, some of which do not necessarily associate physically with this protein. For example, SPARC is thought not to bind to bFGF, but SPARC inhibits bFGF-stimulated endothelial cell cycle progression [70]. Apparently, the effect of SPARC on cell proliferation is complex and could occur through (1) a direct prevention of receptor activation, and/or, (2) a pathway mediated by a cell surface receptor recognizing SPARC, and/or (3) changes in the structure and composition of the ECM that alters mechanical signaling to cells. To date, cell surface receptors for SPARC include stabilin-1 on macrophages and vascular cell adhesion molecule-1 on endothelial cells [74,75]. SPARC also affects integrin engagement on a number of different cell types. Furthermore, SPARC interaction with integrin heterodimers appears to be cell-type dependent [70].

Another significant effect of SPARC on cells in culture is its capacity to elicit changes in cell shape. Many cell types plated on various substrata retract their filopodia and lamellipodia and assume a rounded phenotype after exposure to SPARC. Bovine aortic endothelial cells, for example, are prevented from spreading in the presence of SPARC [70]. Clearly cell rounding could contribute to the inhibition of cell cycle mentioned previously; however, these two effects of SPARC appear to be independent. Motamed and Sage have shown that inhibition of tyrosine kinases reverses the counter-adhesive function of SPARC but has no effect on cell cycle inhibition [76]. Thus at least in endothelial cells, SPARC appears to mediate two aspects of cell behavior through different mechanisms. In support of SPARC as a regulator of cell proliferation, primary mesenchymal cells isolated from SPARC-null mice proliferate faster than their wild-type counterparts [77].

In addition to its effect on cell cycle, evidence shows that SPARC influences TGF-β-dependent pathways in some cell types. Mesangial cells isolated from SPARC-null mice show a decrease in collagen I expression accompanied by a decrease in the levels of the cytokine TGF-β1, in comparison to wild-type cells [78]. TGF-β is a known positive regulator of ECM synthesis. Addition of recombinant SPARC to SPARC-null mesangial cultures restores the levels of collagen I and TGF-β nearly to those of wild-type cells [78]. SPARC also promotes the TGF-β signal transduction pathway in epithelial cells [79]. In pericytes the absence of SPARC increased TGF-β-mediated inhibition of migration, an effect dependent upon the TGF-β receptor endoglin and integrin α_v [80]. Thus it appears that SPARC can act as a regulator of TGF-β activity and, perhaps by extension, represents one mechanism by which SPARC can influence collagen I production.

The targeted inactivation of the SPARC gene in mice has provided insight into SPARC activity in tissues. The majority of SPARC-null phenotypic abnormalities, however, are seen as aberrant ECM assembly. Early onset cataractogenesis reported in two independently generated SPARC-null mice appears to be caused by inappropriate basement membrane production by lens epithelial cells [81]. In the absence of SPARC, collagen IV, a SPARC ligand, is not localized to the outer border of the lens capsule, in contrast to its distribution in wild-type capsules [82].

Levels of fibrillar collagen are reduced in SPARC-null connective tissue, including skin, heart, fat, tendon, and periodontal ligament. In addition, collagen fibrils, as viewed by electron microscopy, are smaller and more uniform in diameter in comparison to those of wild-type tissues. Interestingly, many characteristics of the collagen phenotype in SPARC-null periodontal ligament are reversed through inhibition of transglutaminase [83]. Hence, SPARC is implicated in the formation of collagen cross-links mediated by transglutaminase. Supportive of a critical role of SPARC in collagen deposition, fibrosis in SPARC-null mice is decreased in tissues such as, heart, lung, kidney, liver, and skin [81]. Hence an established body of evidence demonstrates a function of SPARC in assembly and stability of fibrillar collagens and basement membranes. In tissue engineering applications, modulation of SPARC expression is predicted to contribute to ECM remodeling. For example, the inhibition of SPARC expression in pancreatic stellate cells grown as 3D engineered tissue results in robust remodeling of the deposited ECM [84].

Two studies have examined SPARC interaction with collagen I to define the SPARC-binding site(s) on fibrillar collagen heterotrimers [85,86]. Interestingly, SPARC

shares the identical fibrillar collagen−binding site with that of discoidin domain receptor (DDR) 2 and von Willebrand factor. Downstream signaling pathways activated by DDR2 engagement of collagen include increased cell proliferation and enhanced cell migration. Thus engagement of collagen I by SPARC is predicted to prevent collagen engagement by DDR2 (and by von Willebrand factor) and perhaps diminish DDR2-dependent cell activities, such as cell proliferation, in certain cells and tissues.

Once again, we see SPARC as an example of a multifunctional protein able to regulate cell shape and modulate growth factor activity. In addition, SPARC appears to be a key factor in ECM assembly in both basal lamina and connective tissues. Consistent with these functions, a homolog of SPARC termed SPARC-like protein 1 (hevin) is shown to be counter-adhesive and modulatory of ECM structure [68].

Conclusion

In addition to the proteins described here, others are also potential candidates for inclusion in the family of matricellular proteins. These include small proteoglycans, such as the matrix-associated protein and decorin, which has been shown to be an endogenous regulator of TGF-β activity, as well as certain members of the CCN gene family [87]. The CCN proteins are secreted, modular, and exhibit functions based, at least in part, on integrin-mediated mechanisms. While CCN1 (CYR61) enhances apoptosis in fibroblasts, endothelial cell adhesion to CCN1 promotes cell survival [87]. Periostin and other SPARC family members are also recognized as matricellular proteins [68,88]. Further analysis of these proteins and their actions will expand our comprehension of matricellular proteins and the functions they serve in regulating cell interaction with components of their immediate environment.

In any cellular environment, numerous extracellular signals are in place to control cell behavior. In adult tissues, injury and disease both lead to a wide-scale release of multiple factors, either secreted from cells or sequestered in the ECM, that are capable of eliciting potent cellular responses. Appropriately, an increase in the expression of many matricellular proteins is associated with pathological events. Hence, the matricellular proteins appear to be ideally suited to act as modulators of these extracellular signals. They are able to serve as bridges between the ECM and cell surface receptors, such that cell shape changes or cell movements can be initiated prior to matrix breakdown or synthesis. At least three matricellular proteins, thrombospondin-2, osteopontin, and SPARC, appear to participate in ECM deposition, either through the promotion of collagen fibrillogenesis or the enhancement of collagen production. Matricellular proteins can either inhibit or potentiate specific growth factor signal transduction pathways. Thus different growth factor effects may be amplified or subdued by the presence or absence of these proteins. The fact that multiple receptors exist for most of the matricellular proteins allows for diverse functional consequences for different cell types in a complex tissue in response to a matricellular ligand. A given repertoire of cell surface receptors can stimulate (or inhibit) a particular pathway in a cell-type-dependent manner. Apparently, evolution has fine-tuned these proteins to serve as specialized mediators of extracellular signals that provide a coordinated, efficient resolution of tissue injury.

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Chapter 13

Cell and matrix dynamics in branching morphogenesis

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Introduction

Branching morphogenesis is the developmental process by which many organs, including the salivary gland, lung, kidney, and mammary gland, generate tree-like architectures. This extensive branching process enables these developing organs to increase their epithelial surface area greatly to achieve sufficient tissue complexity for mediating secretion, exchange of gases, and excretion essential for life. Generating more branches requires constant remodeling of the extracellular matrix (ECM) [1]. Two cell populations essential for initiating the process of branching and ECM remodeling are epithelial cells and mesenchymal cells. The epithelium generates branches by expanding and repetitively subdividing the epithelium while maintaining stem and progenitor cells [2], and the mesenchyme generally provides the signaling required for the organ to complete the steps of organogenesis. There are, however, also important bidirectional epithelial−mesenchymal inductive signaling interactions during early development in order to initiate and coordinate branching [3−5].

The ECM secreted by mesenchymal and epithelial cells can influence the microenvironment from the earliest stages of bud initiation to the generation of extensive branching using mechanical as well as chemical signaling [6]. The ECM is not only necessary for maintaining structural support for developing organs, but it is also important for cell communication. ECMs can exist in many forms, but two major forms, basement membrane (BM) and interstitial matrix, are critically important during branching morphogenesis. Epithelial cells use the thin, dense, and sheet-like BM as their substrate, the functions of which include providing structural support and elasticity to the epithelium, as well as transmitting mechanical forces and chemical signaling to the tissue [7]. On the other hand, the interstitial matrix maintains and helps regulate spaces between cells, modulating chemical and mechanical communication between cells and within tissues [7].

This chapter will focus on the different ways in which the ECM plays roles in branching morphogenesis of the lung, salivary gland, and kidney as our primary examples for this process. It will also explore how cellular dynamics, the microenvironment, and signaling influence how this process varies between different branching organs. A key emerging principle relevant to tissue engineering is that even though there are similarities between the mechanisms of branching morphogenesis in different organs, there are also many striking differences in the specific details of the cellular mechanisms of tissue morphogenetic remodeling used by each organ, particularly in the specific signaling molecules involved in each type of branching morphogenesis. Consequently, approaches used for regenerative medicine, which are based on insights from developmental biology, may require organ-specific strategies.

The basis of branching morphogenesis

For proper branching morphogenesis, cells need to go through coordinated proliferation, migration, differentiation, and cell death [2]. Epithelial cells of branching organs rearrange to form lobular or tubular structures [8]. These structures can change during different stages of development or in response to physiological changes in some tissues, such as the tubular-to-lobular changes

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observed in the mammary gland during lactation. However, not only do the morphologies of the structures vary between tissues, but the branching patterning itself can also vary between tissues. The branching patterns of lung and trachea are considered to be stereotyped or hardwired [9], because they follow the same predetermined sequence of branch generations (bottlebrush, planar array, and rosette) that are coupled with specific functions (scaffold, edge, and surface/interior) [10]. In addition, the patterns of signaling required to establish the timing, spacing between branches, and the positions of branches with respect to the mother branch are conserved between species.

This is not the case with tissues like the salivary gland or mammary gland. These two glands do not require a specific stereotyped order of steps to trigger branch formation as observed in the lung. In further contrast the kidney exhibits a hybrid phenotype, where it initially appears to be hardwired, but the later branching pattern varies between individual kidneys [11]. During the process of branching morphogenesis, organs create new branches by two events termed budding and clefting [8]. Budding refers to formation of a branch or stalk from the primordial epithelium or from the lateral side of a preexisting branch, while clefting refers to occurs to the splitting of a branch tip by a cleft to generate more tips.

Branching morphogenesis in the lung

As the clearest example of stereotyped or hardwired morphogenesis, the lung utilizes three modes of branching in a repetitive, predetermined series of patterns (domain branching, planar bifurcation, and orthogonal bifurcation) [10]. The repetitive pattern is believed by some to be conserved among different animal species. Others have challenged this concept, suggesting that branching can differ between organisms of the same genetic background, with branches after the first branch being the result of the epithelium reacting to growth of the mesenchyme with the aim of trying to fill spaces [12,13]. However, since both viewpoints consider the initial branches to be stereotyped and conserved among species, the principles of early lung branching can be understood from examining the morphogenesis of the *Drosophila* trachea, which demonstrates the sophisticated types of regulation of signaling needed for branching morphogenesis.

Budding of the *Drosophila* trachea starts when tracheal cells invaginate to form tracheal sacs. Cells around the tracheal sacs release a chemoattractant ligand called fibroblast growth factor (Fgf)/branchless (Bnl) [14]. As portrayed in Fig. 13.1, cells closer to the source of this ligand have higher expression of its receptor [fibroblast growth factor (FGF) receptor (FGFR)], and cells farther away have lower expression due to the decreased

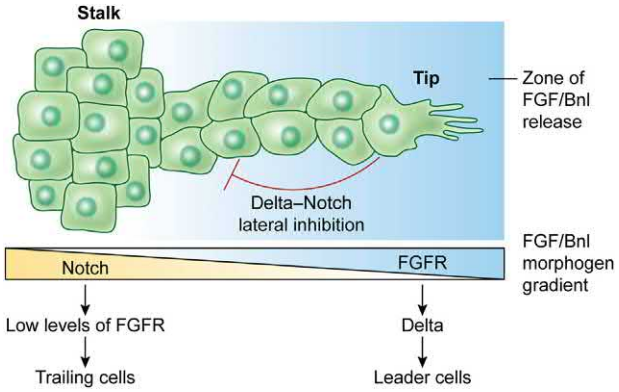

FIGURE 13.1 Budding in the *Drosophila* trachea. During bud initiation, epithelial cells sense the chemoattractant signal from a gradient of FGF ligand released by the mesenchyme. Epithelial cells closer to the site of release extend protrusions that help them monitor and interact physically with the microenvironment. These cells at higher FGF concentrations (closer to the region of FGF release) have higher expression of the FGFR and become leader cells. Higher levels of FGF near the leader cell also activate Delta in these cells, whereas lower levels of FGF activate Notch in the trailing cells. Enhanced expression of Notch leads to the downregulation of FGF signaling to establish trailing cell fate. Once both cell fates are established, there is collective migration of the trailing cells following the leader cells. *FGF*, Fibroblast growth factor; *FGFR*, fibroblast growth factor receptor.

morphogen gradient. Differential expression of Fgfr/breathless (btl) (the mammalian homolog is FGF10) determines cell fate by making the high-expressing cells the leader cells and the low-expressing cells the trailing cells [14–16]. The pattern of FGF10 expression is believed to be controlled by mesenchymal cell expression of patched1 (Ptch1, sonic hedgehog receptor), either directly through hedgehog signaling (Gli3 expression) or indirectly by Forkhead box F1 inhibition [17]. Higher expression of FGF in the tip leads to activation of Delta in the tip and Notch in the stalk. Through a process called Delta–Notch lateral inhibition, Notch activation in the stalk reduces the levels of FGFR, preventing trailing cells from turning into leader cells [8,15,16,18]. Leader cells have long dynamic filopodia at their leading edge that actively pull on the ECM [19], while the trailing cells respond to the pulling by migrating collectively for tissue elongation.

Actomyosin contractility mediates the change in epithelium shape to generate the initial bend for budding, as well as the rearrangement and deformation of the ECM during trailing cell collective migration [20]. Dickkopf (DKK) family members are expressed in the bud tips during development and are known to regulate Wnt/β-catenin signaling in this zone, preventing epithelial differentiation to form a lumen in this region [21]. In addition, DKKs become highly expressed in the zone undergoing clefting in mammals, contributing to the deposition of fibronectin for promoting branching [22].

Actin localization is also site specific. Actin accumulates at the basal membrane and small apical junctions of the leader cells, while the trailing cells only have higher actin on their apical surfaces [18]. In addition, the apical membrane of the leader cells is oriented perpendicular to the direction of branching elongation, while in trailing cells, it is parallel to the elongation [18]. The actin accumulation at the basal membrane suggests that there is a constriction of the dorsal epithelium or epithelial sheets during budding [20,23].

Turning to studies of mammalian lung, after bud initiation, epithelial cells undergo proliferation to increase the size of the tip [24]. The tip becomes flattened before clefting occurs. It has been suggested that in mammalian lung, bone morphogenetic protein (BMP)-Smad1/5/9 signaling helps regulate epithelial proliferation and apoptosis during clefting [25]. BMP4 is expressed in the tip to inhibit supernumerary formation of newer branch tips [26].

As described in Fig. 13.2, accumulation of fibronectin is found in the indentation sites of mammalian lung [27], suggesting that it may help determine the sites of clefting. In addition, the location of branching corresponds to locations of BM thinning [28]. Initiation of clefting in *Drosophila* is known to occur when a tip splits due to the divided localizations of FGF [29], allowing the mesenchyme to physically shape the cleft site. Whether this process occurs in mammalian lung development needs to be clarified. In fact, in mammalian lung development, mesenchymal cells positive for α-smooth muscle actin (α-SMA) expression have been shown to appear in the middle of the flattened epithelium, constricting the epithelium and constraining its expansion to the edges, thereby creating the first bifurcation. Disruption of FGF signaling results in smooth muscle covering the entire epithelium without increasing cell differentiation. In this case, even though the initial cleft can be formed, it cannot proceed to complete bifurcation [30]. These findings suggest that FGF signaling may not only help in shaping the epithelium by its localization but also by regulating smooth muscle growth and differentiation. FGF9 activity mediated through the FGFR1/2 receptor helps to maintain a population of undifferentiated smooth muscle cells and prevent α-SMA localization in the distal mesenchyme [31]. FGF10 allows for the differentiation of distal lung mesenchymal cells into smooth muscle by stimulating BMP4 upregulation in the epithelium [32].

Neuronal innervation in the lung is important for its mechanotransduction, maintaining pressure in the lung by generating muscle contraction and relaxation, which are important for lung growth. Although the first bifurcation will appear at embryonic day (E) 10.5, neurons are still undifferentiated at this point. It is not until E11 that nerves are detected in the smooth muscles. By E13, some of the neuronal projections extend to the mesenchyme, suggesting that there could be some neuronal signaling to the mesenchyme that might help regulate its growth/migration [33].

Once the first cleft occurs in the mammalian lung, the generally accepted stereotyped branching mechanism takes place, starting with domain branching controlled by a proximal-distal and circumferential system, which determines the next position of the branch. Domain branching occurs by budding, allowing the organ to generate new branches on the lateral side of preexisting branches in a

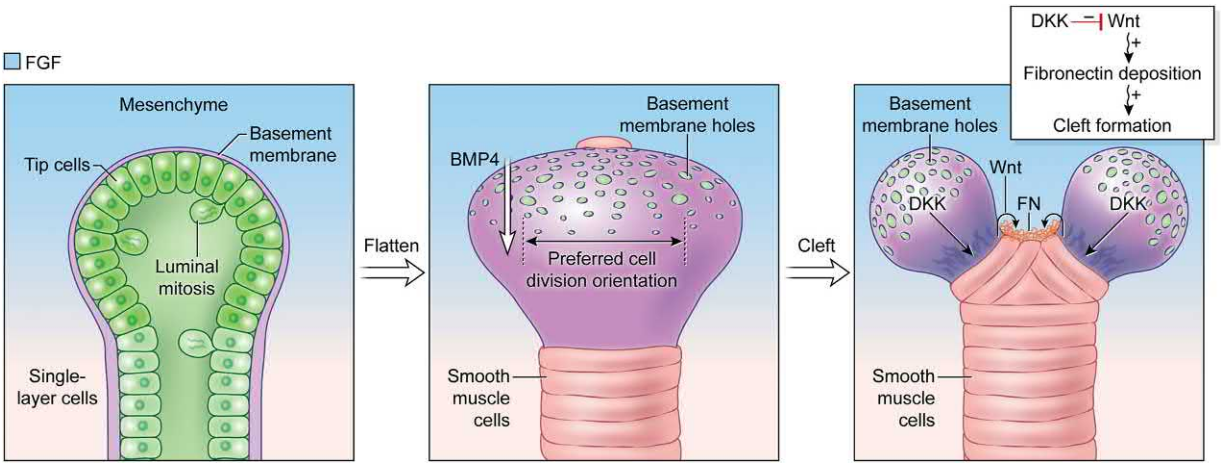

FIGURE 13.2 Cleft formation in the mammalian lung. The lung bud arises from a single layer of epithelial cells that proliferate and flatten prior to clefting. During bud outgrowth the axis becomes flattened and cells divide in parallel around it. BMP4 is expressed in the bud tip, helping to control bud outgrowth. The basement membrane becomes thinner and perforated by microscopic holes, enhancing flexibility for epithelial tip outgrowth and providing epithelial–mesenchymal contact sites. DKK is expressed in the bud tips, which downregulates Wnt signaling and promotes deposit of FN. Smooth muscle then differentiates and extends across the middle of the flattened epithelium, helping to deform the tissue to mediate the bifurcation. *BMP*, Bone morphogenetic protein; *FN*, fibronectin.

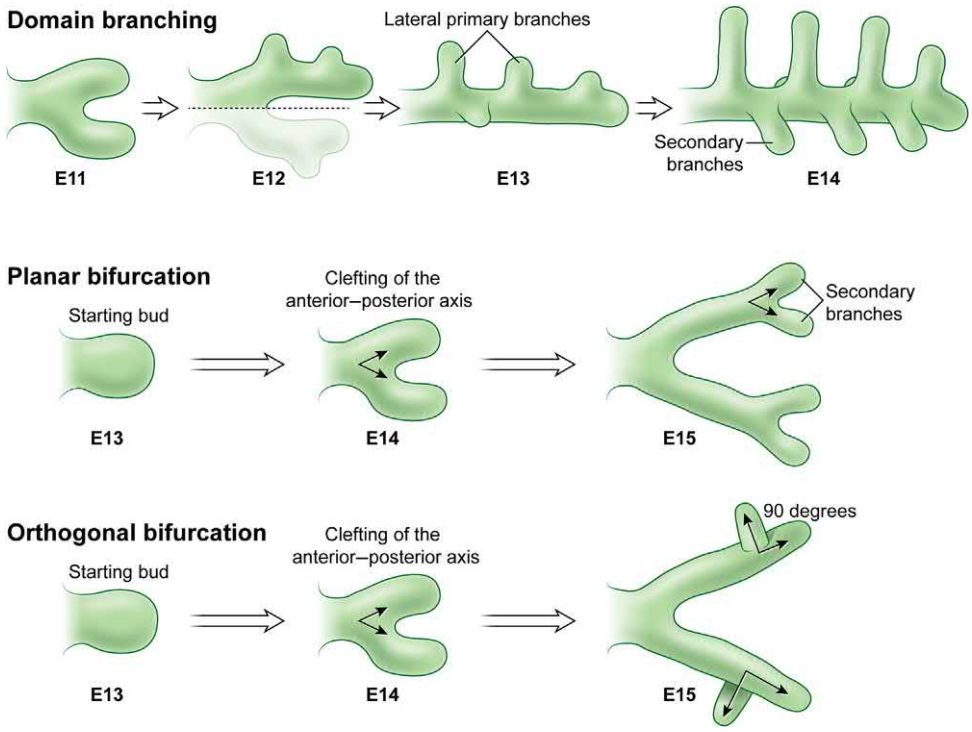

FIGURE 13.3 Branching types in the lung. The lung utilizes three different types of branching to increase its epithelial surface area and achieve complexity. The first type is called domain branching that is observed at approximately E (embryonic day) 12. A branch appears at the lateral surface of the first bifurcation, and its secondary branches appear between the initial parental branches in a proximal-to-distal pattern, thereby generating a structure that resembles a bottle brush. As the lung grows, planar and orthogonal bifurcations are visible at E13. Planar bifurcation generates new branches in a planar array through a series of tip expansions and splits along an anterior−posterior axis. In orthogonal bifurcation the initial branch is similar to that observed in planar bifurcation, except that the tip of a secondary branch is generated at a 90-degree angle (perpendicular) to the parental branch tip, resulting in new branches that face in opposite directions.

bottlebrush configuration. As depicted in Fig. 13.3, domain branching is observable at approximately E12 and continues in later embryonic stages. But as the lung develops further, other types of branching modes appear. One is planar bifurcation, in which there is an expansion of the tip followed by a split (clefting), creating a bifurcation along the anterior−posterior axis and then an additional bifurcation that generates planar arrays. The other mode of branching is orthogonal bifurcation, in which clefting is followed by a 90-degree rotation between rounds of branching to generate rosettes [10], where new branches point in opposite directions (Fig. 13.3). The surface area of the developing lung tissue therefore increases through the continued formation of new branches following a specific, stereotyped sequence of budding and clefting.

Branching morphogenesis in the salivary gland

In contrast to branching in the lung and kidney, salivary gland branching morphogenesis involves mesenchymal cells from the cranial neural crest, which aggregate around the epithelium [34]. Nevertheless, similar to branching in the lung, branch initiation in the submandibular salivary gland (SMG) starts with invagination of the epithelium toward the mesenchyme as a single bud (Fig. 13.4). FGF7/FGFR2b signaling induces widespread epithelial proliferation and budding in the SMG [35,36]. Budding is associated with the formation of a shallow cleft that will progress deep into the initial globular bud to divide it into new buds. The originally narrow clefts widen substantially as they reach the interior of the bud, thereby delineating the secondary duct, which elongates to generate the stalk. These steps of cleft formation, duct elongation, and subsequent lumen formation are essential to generate the final complex epithelial tree-like structure [27,36].

The SMG parasympathetic ganglion (PSG) develops in parallel with growth of the epithelium. The PSG is derived from neural crest cells, which migrate along chorda tympani nerves and differentiate into βIII-tubulin-expressing (TUBB3 +) neurons [37]. PSG innervation produces the neurotransmitter acetylcholine. Acetylcholine release by the ganglion increases transcription of the epidermal

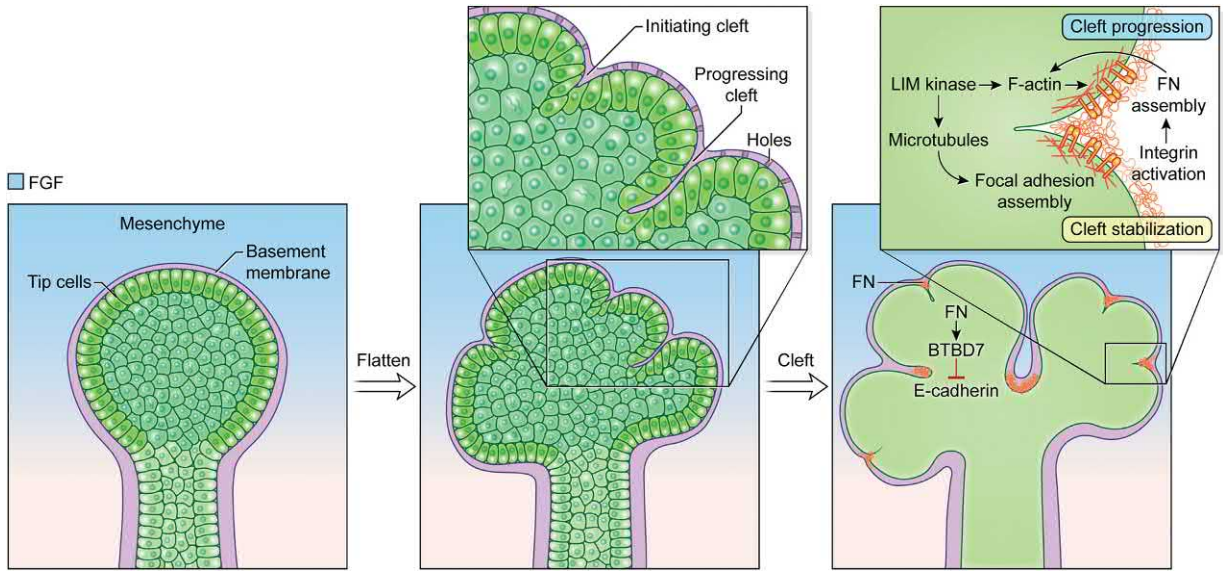

FIGURE 13.4 Cleft formation in the salivary gland. In contrast to the lung and kidney, the salivary gland bud is a multilayered epithelium. The outer tip and inner tip cells have different shapes and speeds of migration/motility, associated with differential BTBD7 expression. To generate a cleft the ECM penetrates the outer epithelial cell layer, with cell separation and insertion of the basement membrane into the bud interior. The basement membrane is thinner at the tip of the bud with microperforations, and it is thicker at the cleft, toward the base of the bud, and along the duct. Fibronectin is assembled by a LIMK-dependent pathway and inhibits E-cadherin through BTBD7 signaling, mediating the conversion of cell−cell adhesion to cell−matrix adhesion in the progressing cleft. Stabilization of the cleft is mediated by LIMK, which stimulates focal adhesion through stabilization of actin filaments and microtubule dynamics. *LIMK*, LIM kinase.

growth factor (EGF) receptor (EGFR), which results in increased cell proliferation [38]. Although the difference between salivary gland stem and progenitor cells is not yet clear [34], maintaining an undifferentiated population of epithelial cells is essential for formation of the correct branching pattern. This population of progenitor cells characteristically expresses keratin 5, a cytoskeletal protein. These K5-positive (K5 +) progenitor cells are located in both the SMG end bud and duct during development. K5 + progenitor cells are known to express Wnt. Wnt signaling from K5 + progenitor cells within the duct drives ductal differentiation and promotes gangliogenesis [37] by promoting neuronal proliferation and survival, as well as PSG formation and innervation [34]. K5 + progenitor cells are also present in the end buds, where Wnt signaling is suppressed by FGFR (Fig. 13.5). This Wnt signaling is negatively regulated within the epithelium by FGFR2b/mitogen-activated protein kinase (MAPK) signaling and is stimulated by neuregulin Erb-B2 receptor tyrosine kinase 2 (ErbB2/3)/phosphoinositide 3-kinase (PI3K)-dependent signaling [34]. The negative regulation via FGFR maintains the K5 + progenitor cells in the end bud and inhibits ductal fate and lumen formation [39]. Carbachol, an acetylcholine analog, can work together with heparin-binding EGF-like growth factor signaling to increase the proliferation of cells that are not K5 + positive, stimulating lumen formation [38].

The SMG epithelium maintains the PSG through production of the neurotrophic factor neurturin (NRTN). NRTN maintains functional innervation by promoting axonal outgrowth and expression of genes that are important for parasympathetic function. This PSG axonal outgrowth in turn influences morphogenesis by conserving cell−cell adhesion through stabilizing E-cadherin signaling in the end bud and maintaining proper neurotransmitter release [40]. If the PSG pattern is disrupted, it can affect branching morphogenesis by reducing the number of branches that are formed [38].

To complete the process of clefting, different cellular processes occur, including proliferation, cell−matrix adhesion, cell−cell adhesion, and migration [34]. During clefting of the salivary gland and other branched organs, fibronectin is expressed in the BM near the mesenchymal cells and epithelial cells that undergo clefting (Fig. 13.4). This expression of fibronectin can decrease the expression of E-cadherin, a cell−cell adhesion protein—a process believed to occur through the induction of the novel regulator BTBD7 [6,34,41]. BTBD7 has been shown to be expressed near the cleft and to be necessary for regulating the outer layer of highly motile semi-columnar epithelial cells during clefting [41] and has been reported to be essential for effective branching morphogenesis of salivary gland, lung, and kidney. BTBD7 induces the expression of Snail2, which contributes to loss of E-cadherin, though it may also mediate ubiquitination involved in E-cadherin degradation [6]. This mechanism affects the cleft epithelial cells that convert cell−cell adhesions to cell−matrix (especially BM) adhesion during clefting

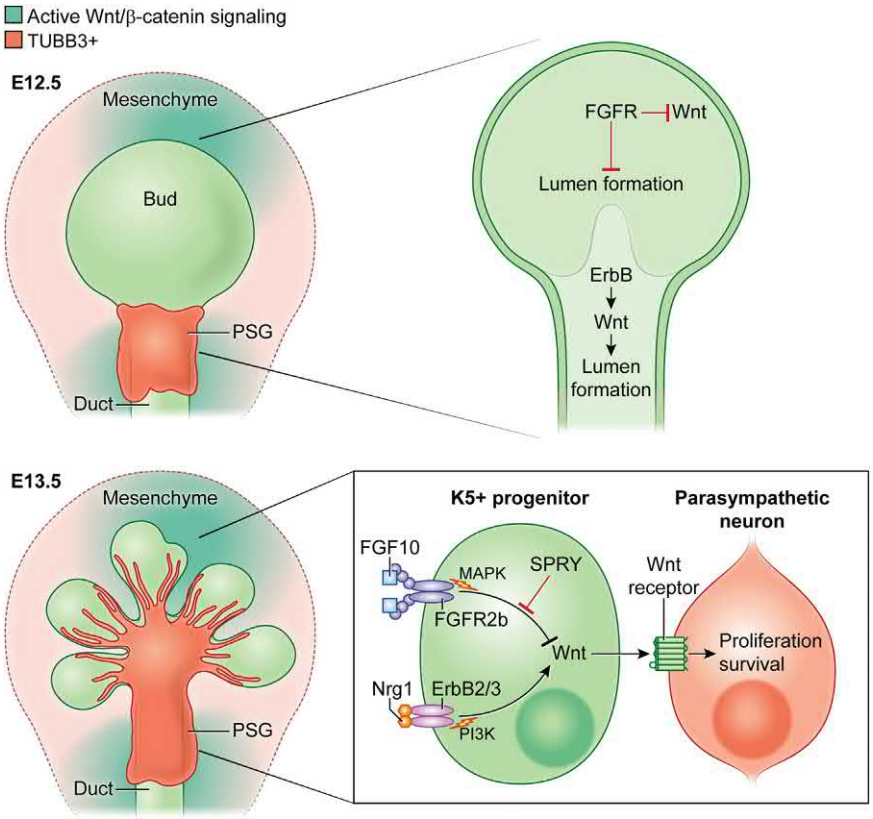

FIGURE 13.5 PSG development in the salivary gland. The PSG grows in parallel with growth of the epithelium. Wnt signaling is one of the mechanisms important for maintaining PSG nerve innervation. Wnt signaling mainly occurs in the mesenchyme near the bud tip and particularly in the ductal area. FGFR signaling inhibits Wnt signaling through downstream signaling that activates MAPK in the bud tip to prevent epithelial differentiation. In the duct, however, this signaling is inhibited by Sprouty (SPRY). Nevertheless, ErbB eventually upregulates Wnt expression in the duct, allowing cells to differentiate to form the lumen by downstream activation of PI3K. This same activation helps promote neuronal proliferation and association with the ductal epithelium. *FGFR*, Fibroblast growth factor receptor; *MAPK*, mitogen-activated protein kinase; *PSG*, parasympathetic ganglion.

[27,41]. In addition to this conversion, cytoskeletal contraction is a vital process for the progression of clefts. Rho-associated coiled-coil containing kinase (ROCK) regulates cytoskeletal contraction, while LIM kinase helps to regulate both actin filaments and microtubules to mediate cleft stabilization. Together these allow progression and proliferation of the cleft to continue [42–44]. Although proliferation plus cytoskeletal and ECM remodeling [34] are known to occur during clefting, in the salivary gland the first branch can still proceed without cell proliferation [8,45,46].

Expanding knowledge of the mechanisms of branching morphogenesis is now being applied to tissue engineering of salivary glands to maintain or restore function. For example, cell surface expression of c-Kit and other stem cell markers was used to isolate stem and progenitor cells from explanted salivary gland cells that had been expanded in vitro into small organoids termed salispheres. These isolated stem cells could be transplanted into the salivary glands of mice that had been X-ray irradiated to mimic the damage from clinical therapy for head and neck cancer. The transplanted stem cells could at least partially restore both salivary gland morphology and secretory function [47,48]. Furthermore, identification of growth factors that can stimulate salivary gland branching in explant cultures has provided a list of candidates that could promote gland stem cell protection or restoration after X-ray irradiation. Successful examples of the latter approach include treatment with keratinocyte growth factor, granulocyte-colony stimulating factor (G-CSF), or neurturin [49–51].

An alternative approach to cell-based tissue engineering for salivary gland regenerative medicine is to take advantage of the previously demonstrated capacity of embryonic salivary gland cells to self-aggregate and initiate budding morphogenesis [52]. Functional regeneration of mouse salivary gland function has been reported using dissociated and self-organized embryonic cells and even embryonic stem cells [53,54]. Notably, identification of two key transcription factors for normal salivary gland development, Sox9 and Foxc1, permitted a novel approach—infection of embryonic stem cells in vitro with adenoviruses expressing Sox9 and Foxc1 to force expression of these factors. When the authors incubated these treated cells with a cocktail of growth factors, they reported successful restoration of salivary gland secretory function in mice [54].

Branching morphogenesis in the kidney

The epithelial bud that initiates kidney branching arises during E10.5–11 in mice [55]. Before this ureteric bud

formation the edge of the epithelial tube needs to become pseudostratified to allow for rapid outgrowth in this region [56]. Interactions between the epithelium and the mesenchyme allow the cells to undergo clefting that occurs at E11.5. The complex tree structure arises by interactions at both gene and protein levels controlled primarily by the Ret/Gdnf, Wnt, and FGF signaling pathways. To initiate the process of branching the mesenchyme divides into two populations: one becomes the cap mesenchyme, while the other forms the stromal cells [57]. Pax2 protein is then highly expressed in the cap mesenchyme and in the epithelial bud. Pax2 positively regulates glial cell–derived neurotrophic factor (GDNF) that is then expressed in the cap mesenchyme, and its receptor GPI-linked protein (GFRα1) is expressed in both the mesenchyme and ureteric bud tip. GDNF expression activates the catalytic domain of the receptor tyrosine kinase RET in epithelial cells, helping guide the invasion and outgrowth of these cells into the mesenchyme (Fig. 13.6).

Budding in the kidney arises from a tubular outgrowth of the Wolffian duct [58]. To establish the bud tip, cells compete with each other, and the cell expressing the highest level of Ret becomes the tip [59,60]. Ret expression helps to maintain high levels of Sprouty1 in the Wolffian duct to prevent an excess of cells from becoming leader cells. Sprouty1 mediates this action by antagonizing GDNF/RET signaling in the duct [61]. BMP4 and transforming growth factor beta (TGF-β) have been shown to inhibit bud formation [62,63]. In the kidney, Gremlin 1 (GREM1) reduces the expression of BMP4 to allow budding to proceed.

The ligand–receptor pair GDNF/RET contributes to epithelial bud proliferation, cell survival, and branching of the epithelium. Although the mechanism is not yet entirely clear, some of the genes that are regulated by GDNF activation include two members of the Pea3 family of ETS transcription factors, Etv4 (Pea3) and Etv5 (Erm) [58]. Etv4 and Etv5 are expressed in the bud tips and have been shown to target Myb—in other organs, these transcription factors are known to promote the proliferation of progenitor cells and inhibit differentiation. Consequently, Myb signaling could be a mechanism used to maintain a population progenitor cells during clefting events. Similar to lung branching, in order for clefting to occur in the kidney, proliferation needs to occur at the tip, followed by deformation and tip-splitting events [8]. This deformation occurs as the bud tip, composed of a single layer of mitotic cells, undergoes a sequence of enlargement, flattening, and extension in opposite directions before splitting [64]. During cell proliferation, the premitotic cells separate from the single-layer ureteric tip and complete their cell division in the lumen before integrating back into the site of origin—a process termed mitosis-

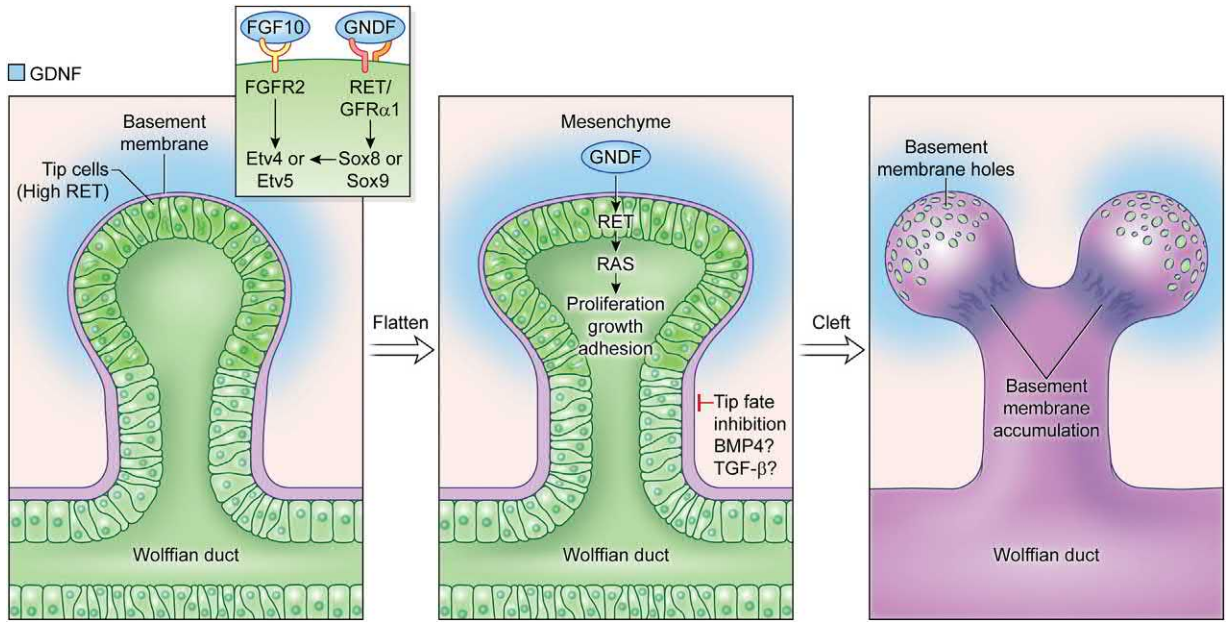

FIGURE 13.6 Cleft formation in the kidney. In the kidney the outer epithelial cells of the tip have higher expression of the Ret receptor than their proximal counterparts. GDNF is expressed in the cap mesenchyme and acts in parallel to FGF10 signaling to activate Etv4, which helps guide the migration of nephric duct cells with high Ret expression from the Wolffian duct to the site of the outgrowth to generate the tip of the first bud. For clefting to occur the pseudostratified tip flattens and proliferation increases due to the activation of Ras. It is believed that BMP4 and TGF-β are involved in preventing the trailing cells from becoming tips, and that they instead promote duct formation. *BMP*, Bone morphogenetic protein; *FGF*, fibroblast growth factor; *GDNF*, glial cell–derived neurotrophic factor.

associated cell dispersal [65]. Furthermore, a cell tracking study showed that during clefting of Ret-MADM6 clones, cells that have high levels of Ret and Etv4 remain closer to the tip compared to cells with lower expression, suggesting that this signaling guides cell movement at the tip [60].

Degradation of ECM components by matrix metalloproteinases is necessary for ECM remodeling to permit proper branching. Etv4 can transactivate the protease matrix metalloproteinase-14 (Mmp14). Mmp14$^{-/-}$ mice have kidney branching defects, and Etv4$^{-/-}$ and Etv5$^{-/-}$ mutants show a reduction in MMP14, suggesting that one of the roles of these transcription factors is to regulate ECM remodeling by maintaining appropriate levels of MMP14 [58]. In addition, the Met tyrosine kinase receptor is present in both the bud tip and mesenchyme, and it becomes downregulated upon Etv4 inhibition. Hepatocyte growth factor (HGF), which is expressed in the mesenchyme, is known to bind to Met and to activate downstream signaling important for cytoskeletal dynamics, focal adhesion, and cell−cell junction reshaping [66]. Both the EGFR and HGF have been implicated in kidney tubulogenesis. However, knockout of Met and HGF genes in mice does not reveal a significant reduction in branching. Nevertheless, Met$^{fl/fl}$ mutants have increased expression of EGFR, which suggests that HGF and EGFR may cooperate, and that the upregulation of EGFR could occur to maintain tissue homeostasis [66]. Other studies have shown that GDNF-induced Etv4 will activate Met in motor neurons and HGF in the mesenchyme can further induce Etv4. HGF is known to promote axonal growth and survival of motor neurons, which could occur by recruiting more motor neurons to the Etv4^{+} population [67]. Other signaling, such as Wnt-1 is also known to induce Etv4. In kidney, nerve innervation is linked with vascularization, suggesting that both are controlled by the same mechanism [68]. Consequently, Etv4 might be a mechanism by which nerve innervation occurs in the kidney.

Stalk elongation in the kidney occurs by convergent extension that is dependent on Wnt and planar cell polarity (PCP) signaling [69]. PCP promotes stalk elongation by orienting cell division [70].

Although the kidney does not follow a strict stereotyped branching pattern as in the lung, and the angles of the branches are not conserved, the extent of elaboration of "clades" or "lobes" is conserved and comparable between kidneys from organisms with the same genetic background. Branch growth between kidneys in embryos is similar at early stages with a shape that is relatively lobular with a bulging tip connected to short branches, but after E15.5, the similarities are no longer conserved. At E18.5, the branches become elongated and acquire a tubular shape [8]. Taken together, this information indicates that in later branching events, the kidney does not follow the stereotyped pattern of branch formation during development as described in the lung, since its pattern is not reproducible across developmental time. That is, by not following a strict stereotyped branching program, individual kidney branch points and branch shapes are not comparable between organs from different embryos at the same developmental stage [57]. Since even different mouse embryos display kidney shape differences, it is no surprise that there are differences between mouse and human kidneys at both structural and developmental levels.

Contributions of other cell types

As indicated in previous sections, epithelial and mesenchymal cells are not the only important contributors to branching morphogenesis. Neuronal innervation, smooth muscle, and blood vessels are examples of other cell populations that also play critical roles in achieving successful branching morphogenesis.

The interaction of the salivary gland PSG and epithelium is an excellent example of how two cell populations interact to promote development of each other. In salivary gland development the PSG maintains K5 + progenitor cells by acetylcholine and EGF signaling [38]. In contrast to the lung, the neuronal population in salivary gland can be observed even during formation of the initial bud. The PSG helps to develop the ductal epithelium by promoting lumen formation and ductal elongation via vasoactive intestinal peptide (VIP) signaling. Inhibition of the VIP receptor in salivary gland leads to an increase in ductal width correlated with an increase in tight junctions along the midline rather than toward the bud tip as the bud undergoes branching. In addition, glands in which VIP is inhibited exhibit impaired localization of nerve innervation. Increasing VIP expression results in increased concentration of tight junctions along the midline of buds undergoing clefting and an increase in nerve innervation around the duct relative to controls [71]. In the lung, innervation does not appear until branching has started [33,37], suggesting that it is important for the formation of subsequent branches but not for the first bifurcation.

Blood vessels have been shown to be important for mediating the stereotyped program of branching characterizing embryonic lungs. Inhibition of vascular signaling by a vascular endothelial growth factor (VEGF) decoy receptor decreases the angle of rotation of dorsal-ventral branching during domain branching from 90 to 40 degrees and reduces the number and length of such branches. However, there is a concomitant increase of ectopic branches, which affects the overall architecture of the lung and generates a flattened organ. Restoration of VEGF signaling after blood vessel inhibition results in a

similar phenotype rather than rescuing normal branching, suggesting that the physical presence of blood vessels is crucial and that it is not altered VEGF signaling that causes disruption of the stereotypic branching pattern in the lung [72].

In the pancreas, another branched organ, blood vessels do not contribute to branching as they do in the lung. The removal of endothelial cells or inhibition of VEGFR2 instead increases organ size and contributes to cell differentiation. Conversely, increasing VEGF signaling leads to a reduction of branching and overall pancreas growth. In addition, it leads to undifferentiated trunk epithelium, suggesting that the function of vascular endothelium in the pancreas is to maintain a population of undifferentiated epithelial cells during branching [73,74].

The nonstereotypic, relatively stochastic mode of branching of salivary glands is also modulated by endothelial signaling without blood perfusion [75]. Branching in this developing organ is inhibited by inhibition of VEGF signaling that reduces the number of progenitor cells and increases duct differentiation markers. Addition of exogenous growth factors (IGFBP2 and IGFBP3) can partially rescue branching, suggesting that endothelial cells may stimulate neighboring mesenchyme cells to produce these growth factors to modulate the differentiated phenotype and branching of the salivary epithelium.

MicroRNAs in branching morphogenesis

MicroRNAs are small (~22 nucleotides long) noncoding RNA transcripts that regulate numerous targets through binding to the 3'untranslated regions (UTRs) of target mRNAs and affecting protein production by interfering with mRNA translation. MicroRNAs can function locally by binding to a target mRNA within a specific group of cells or globally via extracellular vesicle delivery to affect multiple tissues throughout the body. MicroRNAs are important for maintaining homeostasis within tissues, but they have also emerged as important players during embryonic development. Dicer, an endoribonuclease that is required for correct microRNA processing, can be detected in the distal mesenchyme and epithelium, zones of high proliferation, and branching in development [76] and has been shown to be critical for branching morphogenesis, proper epithelial development, and angiogenesis. Dicer-null mutants show epithelium detachment from the mesenchyme, dilated branching tips, and fewer branches compared with controls. In addition, inactivation of Dicer causes an increase in FGF10 expression in the mesenchyme, which in turn affects other genes that are essential for lung branching [77]. During branching morphogenesis the location of FGF10 expression changes to stimulate the outgrowth of the bud, which does not occur in the Dicer-null mutant. The lack of a normal pattern of FGF10 expression results in arrest of new branch formation. However, which Dicer-dependent microRNA or microRNAs regulate the specificity of FGF10 location during branching morphogenesis remains unknown.

miR-17, miR-20a, and miR-106b have been shown to be important during lung branching morphogenesis. These three microRNAs are required for the regulation of mitogen-activated protein kinase 14 (Mapk14) and signal transducer and activator of transcription 3 (Stat3). Activation of both Mapk14 and Stat3 are important for maintaining E-cadherin expression levels needed for cell−cell adhesion. Downregulation of these microRNAs results in an abnormal branching pattern in lung explants with an increased number of terminal branches superimposed on one another, affecting the overall branching pattern [78]. There is also evidence that other microRNAs, such as miR-221 (antiangiogenic) and miR-130a (proangiogenic), influence airway branching by regulating vascular density. Alterations in these microRNAs alter vascular density by disrupting the communication between endothelial and epithelial cells, which in turn affects vascular patterning—but it also influences branching, with high expression of proangiogenic signaling resulting in an increase in branching and elevated expression of antiangiogenic signaling resulting in decreased branching [79].

Dicer has also been shown to be important for the maintenance of nephron progenitor cells and proper branching in kidneys. Dicer depletion results in branching deficiencies, including an absence of branching tips in the renal cortex correlated with a reduction of Wnt11 and c-Ret at the bud tip. In addition, inactivation of Dicer in renal Foxd1 + stromal progenitor cells disrupts elongation and segmentation of the nephron [80]. Among the microRNAs affected by Dicer inactivation, seven have been implicated in migration, proliferation, and activation of Wnt signaling. Loss of Wnt expression due to inactivation of Dicer leads to a reduction in ureteric bud branching [81].

Similar to the lung and kidney, microRNAs in the salivary gland, such as miR-21 and the miR-200c family, have been shown to play important roles during branching morphogenesis. miR-21 expression in the submandibular mesenchyme during branching morphogenesis results in the activation of MMPs, causing degradation of the ECM through downregulation of reversion-inducing cysteine-rich protein with Kazal motifs (Reck) and programed cell death 4 (Pdcd4) in the mesenchyme [82]. On the other hand, miR-200c expression in the epithelial end bud is important for the regulation of FGFR-dependent epithelial proliferation via targeting the very low-density lipoprotein receptor (Vldlr), its ligand reelin, and potentially via heparan sulfate (HS) modification [83]. In addition, there is evidence that microRNAs can affect branching locally

through local epithelial expression of miR-200c and globally via microRNAs expressed in mesenchymal cells that can regulate targets in epithelial cells through export of microvesicles. miR-133b has been found to be produced in the mesenchyme and delivered to epithelium. It has been suggested that miR-133b regulates end bud morphogenesis by targeting disco-interacting protein 2 homolog b (Dip2b), allowing end bud progenitor proliferation [84]. Although previous studies have shed some light on the different mechanisms by which microRNAs may regulate branching, many questions remain unanswered.

MicroRNA involvement in branching morphogenesis is an area of research that is understudied at the moment. We do not yet have a clear understanding of which specific microRNAs regulate transcripts that are essential for branching, how microRNA expression profiles in different cell populations affect cell dynamics, mechanical forces, and interactions with the ECM, nor how these interactions can result in embryonic lethality, organ failure, or disease later in life. MicroRNAs have the capacity to target multiple genes, making them an attractive tool for regenerative and disease therapy [34]. And a single mRNA transcript can be regulated by more than one microRNA, so understanding the many-to-many nature of microRNA regulation is the key to harnessing microRNAs for therapeutic applications.

Extracellular matrix components in branching morphogenesis

The BM is known to provide tissue separation and boundaries during development [7,85]. It is important not only for maintaining tissue boundaries and serving as a substrate for epithelial cells but also for providing biochemical and potential mechanical signals during branching morphogenesis. The BM is mainly composed of laminin, collagen IV, and proteoglycans, often in close spatial association with fibronectin. In this section, we discuss examples of how each of these components participates in branching morphogenesis.

Laminin

Laminins comprise the most abundant glycoproteins of the BM and have important roles in branching morphogenesis. Two key laminins are laminin 1 (now termed laminin-111) and laminins containing the α5 subunit (laminin 511 and laminin 521). Laminin expression correlates with epithelial development [86,87]. In salivary glands, laminin α1 decreases during development, while levels of α5 rise [88] The interaction of laminin γ1 and nidogen in the BM is important for epithelial−mesenchymal interactions during salivary gland branching morphogenesis. Antibody perturbation of the nidogen-binding site substantially reduced salivary gland bud number [89] and the reduction depends on antibody affinity to the binding site. Abnormal growth was observed in the treated glands, characterized by the absence of globular tips. These effects of disrupting the laminin−nidogen interaction could be partially rescued by the addition of exogenous EGF. Since EGF is important for nidogen synthesis, the rescue might result from increased levels of nidogen able to bind to laminin γ1 [89]. In addition, mesenchyme-free salivary gland epithelium has been shown to grow in laminin-1 nidogen gels supplemented with EGF and horse serum at rates comparable to cultures in full Matrigel with the same supplements. Although more collagen IV accumulates with epithelial cells cultured in Matrigel compared to laminin-1 nidogen gels, morphogenesis is comparable, suggesting that laminin−nidogen binding alone can support branching morphogenesis [90].

3-D cultures of pancreas treated with antibodies against laminin-1 display inhibition of ductal formation, indicating that this glycoprotein is also important for pancreatic epithelial cell differentiation to form ducts [91]. In kidney, laminin-1-deficient mice exhibit reduced ureteric branching, with a major reduction of apical tight junctions and defective BMs. Fewer branches are produced due to inhibition of ductal cell proliferation, and sometimes no ducts are produced [92]. Loss of laminin α5 can also result in defective branching morphogenesis of salivary gland, lung, and kidney [87], which indicates that the functions of different laminins in branching do not fully overlap—each has its own specific role.

Collagen

The two largest classes of ECM are the interstitial matrix and BM—each class contains specific collagen types. The interstitial matrix is composed primarily of fibrillar collagen I, important for structuring the physical spaces between cells and helping to modulate cell communication within tissues. In contrast, a major protein of BM is nonfibrillar collagen IV; BMs provide support for epithelial cells, as well as chemical and mechanical cues to the tissue [7,93,94].

In salivary gland, collagens I and III accumulate at the interlobular cleft, along with the collagen IV associated with the cleft BM [1,27,95]. Collagens I and III fibers are observed in the interlobular cleft before new branches are formed [45], suggesting that they may be important for stabilizing the interlobular cleft as the new branch forms [6,96−98].

In salivary gland epithelial explant cultures, treatment with collagenase to disrupt all collagen types results in a basal lamina that is either irregular or sparse with a decrease in collagen fibrils at the epithelial−mesenchymal

interface, and it abolishes branching [95,98]. The effects are reversible, since removal of the collagenase partially rescues gland branching [98]. In lung and kidney cultures treated with collagenase, branching is similarly abolished, and both types of embryonic organ cultures become flattened in shape. The collagenase effects are reversible here as well, since after reincubation with their mesechymes, lung and kidney branching is rescued [99].

Lungs or salivary glands treated with β-aminopropionitrile that inhibits extracellular collagen cross-linking do not show significant differences in branching compared to controls. Nevertheless, treatment with α,α′-dipyridyl or L-azetidine-2-carboxylic acid (LACA), which disrupts collagen synthesis and secretion, inhibits the generation of new branches; in addition, preexisting clefts in both lung and salivary gland fuse together, suggesting that collagen is necessary to stabilize the clefts. These findings suggest that collagen synthesis and secretion are important for branching morphogenesis, but collagen cross-linking is not [100].

The effects of collagenase or removal of mesoderm in reducing branching or flattening organ morphology are not found in development of the pancreas [99]. This suggests that not all branching organs require the same type of mesoderm- and collagen-dependent signaling for branching. Consequently, just as there is specificity in terms of types of growth factor signaling, there are also organ-specific requirements (or not) for these other developmental regulatory factors.

In salivary gland, opposite to the effects of collagenase, inhibition of endogenous collagenases increases bud number by enhancing cleft formation in the glands, accompanied by an increase in collagens I and III accumulation in the cleft and at the epithelial−mesenchymal interface [95,101,102]. The authors concluded that interstitial collagens I and III are essential to support branching either through structural support and/or serving as a scaffold at the epithelial−mesenchymal interface. However, it would be valuable to compare the effects of collagenase and collagenase inhibitors on levels of collagen IV, which was not examined in the original studies.

Collagen IV is thought to play a role in branching morphogenesis by providing structural support in the BM. However, as will be discussed in detail later, the collagen IV/BM at the bud tips needs to be thinned and perforated to permit bud expansion. Treating salivary glands with batimastat, a matrix metalloproteinase inhibitor, results in enhanced collagen IV accumulation at the end buds and reduced bud outgrowth. This treatment also inhibits formation of microperforations in the BM, which are probably necessary for epithelial−mesenchymal contacts during branching. In fact, the treated BM exhibits a reduction of distensibility and restricted epithelial extension. In addition, treating branching salivary glands with the myosin II ATPase inhibitor blebbistatin also results in accumulation of collagen IV and reduction of bud outgrowth, suggesting that both myosin II contractile function and collagen density are important during the remodeling of the BM to form microperforations and permit bud outgrowth [45]. Collagen IV proteolysis can also stimulate bud outgrowth by promoting cell proliferation. The protease-generated noncollagenous 1 (NC1) domain can stimulate bud outgrowth as a result of collagen degradation and release by endogenous membrane-type matrix metalloproteinase-2 (MT2-MMP) [103].

Collagen XVIII has also been shown to be an important player in lung and kidney branching. Collagen XVIII is suggested to act as a HS proteoglycan that regulates binding and release of growth factors at the BM. During branch initiation, collagen XVIII is expressed at the bud tips in lung but at the stalk in kidney. In a heterotypic recombination experiment in which ureteric epithelium was cultured with lung mesenchyme, kidney cells expressed collagen XVIII at the bud tips. However, a similar result was not obtained when lung epithelium was cultured with ureteric mesenchyme, as no induction of branching or altered collagen XVIII expression pattern was observed. These findings suggest that collagen XVIII may contribute to the stereotyped, deterministic signaling and branching observed in the lung [104].

Heparan sulfate proteoglycan

HS proteoglycan (perlecan) has a key function in the BM involving the regulated of release growth factors by binding and releasing them in specific patterns. HS plays critical roles in branching morphogenesis of salivary gland, lung, and kidney [2]. In salivary gland, HS has been shown to increase the binding affinity of FGF to FGFR and to increase FGF concentration in the ECM. HS works cooperatively with growth factors to shape the epithelium. Treating SMG with FGF10 alone leads to formation of ducts with thin end buds. In contrast, treatment with FGF10 plus HS results in ductal growth with wider end buds due to increased proliferation at the tip of the bud [105]. Therefore the HS−FGF−FGFR interaction is important for embryonic salivary gland proliferation and bud shape.

In mesenchyme-free lung epithelium cultures in Matrigel, treatment with FGF10 and sodium chlorate (an inhibitor of sulfation) affects the HS expression gradient and FGF10 local activation. The result is reduced expression of BMP4 in the bud tips and abolition of bud formation. Attempted rescue by treatment with O-sulfated heparins increased levels of BMP4 but failed to rescue lung budding, suggesting that even though O-sulfated HS is important for activation of FGF10, it is not the only player required for bud formation [106]. In kidney,

disruption of HS sulfation also results in reduction of branches and epithelial buds in a dose-dependent manner. Addition of excess exogenous sulfate with the chlorate inhibitor partially rescues the phenotype [107].

Fibronectin and integrins

Fibronectin is located in interstitial matrix and adjacent to BMs and plays key roles in the regulation of cell adhesion and migration. During branching morphogenesis, fibronectin concentrations are high at the BM and near cells undergoing clefting, associated with similar accumulations of BM at these sites; both fibronectin and BM move down into forming and progressing clefts [45,108].

Fibronectin is thought to contribute to the conversion of cell−cell adhesions to cell−matrix interactions by inhibiting E-cadherin via induction of BTBD7 [109,110]. The epithelial cells interacting with the BM at the periphery of buds during clefting appear more activated in terms of speed compared to cells in the bud interior [111]. In addition, when cells interact with the BM, there is an observed increase of fibronectin localization in these regions, suggesting that they can contribute to fibronectin deposition in the clefting sites [108]. In addition to epithelial cells and Wnt signaling, focal adhesion kinase (FAK) has also been shown to contribute to fibronectin deposition within the salivary gland cleft. Inhibition of FAK leads to a disorganized assembly of the BM, disrupting cleft formation and progression [112]. Myosin II is also known to be important, and its inhibition disrupts cleft extension and alters F-actin organization [112].

Inhibition of fibronectin in developing salivary glands results in nearly complete ablation of bud formation with an enlargement of the epithelial stalk; conversely, exogenous fibronectin can stimulate branching [27]. Fibronectin can be regulated by Wnt signaling. In lung branching morphogenesis, expression of the DKK family in bud tips was shown to block Wnt/β-catenin activity in the bud tips. Consistent with a role for Wnt signaling in promoting fibronectin accumulation at the cleft site, lung explants treated with the Wnt inhibitor DKK1 exhibit a decrease in cleft formation, reduction in branching, and enlargement of end buds, all of which are also observed after fibronectin inhibition [21]. In the kidney, fibronectin expression is observed in the ureteric bud with expression evenly between the tip and the stalk. However, fibronectin expression decreases during kidney maturation. Treating ureteric cultures with fibronectin results in an increase in cell migration and cellular elongation in a concentration-dependent manner and treating ureteric cultures with antibodies against integrin $\alpha_3\beta_1$ partially inhibits these fibronectin-dependent effects [113].

At the cell morphological level, changes in cell shape and migration are observed at sites of salivary cell−matrix contacts. The cells closest to the BM, the outer bud cells, have a columnar shape but are more motile, while inner cells have a more rounded shape. The outer bud cells exhibit high affinity for the BM, yet they are also highly motile as they translocate along its surface. When these cells leave the BM during mitosis (which often takes place in the bud interior), they change from columnar to rounded, but they eventually return to the BM and assume a more columnar, laterally motile phenotype. These associated velocity and shape changes comprise a dynamic process.

In the outer bud cells, E-cadherin is located at the cell−cell junctions but not at the regions of the plasma membrane in close contact with the BM. In contrast, E-cadherin is located around the periphery of the inner bud cells. Inhibition of $\alpha_6\beta_1$ integrins in the outer bud cells causes disruptions in interactions with the BM, slows lateral migration, and alters E-cadherin localization to mimic more closely the pattern observed in the inner bud cells. However, the inhibition of $\alpha_6\beta_1$ integrins showed no significant changes in the inner bud cells (which are not interacting with ECM), indicating that integrins help regulate velocity and shape in just the outer bud cells as they interact with the BM. Interestingly, inhibition of E-cadherin in outer bud cells has no major effect, whereas inner bud cells show a loss of cell−cell adhesion with an increase in speed, indicating that E-cadherin is important for stabilizing inner bud cells by maintaining cell−cell adhesions [111]. Similarly, in the developing pancreas, changes in cell shape and increases in cell displacement are observed in the cells that interact with the BM, while the other cells maintain a stable shape. Inhibiting cell−ECM interactions in pancreas explants inhibits branching morphogenesis: Deletion of the β1 integrin subunit disturbs cell−ECM interactions and affects ECM remodeling as indicated by a reduction in BM thickness. In addition, deletion of the β1 integrin subunit changes the characteristic F-actin alignment orientation from perpendicular to parallel [114]. Taken together, these findings point to the importance of a variety of ECM−integrin interactions and functions in branching morphogenesis of multiple organs.

Basement membrane microperforations

The BM provides the substrate under epithelia and separates epithelia from embryonic mesenchyme during development. The thickness of the BM changes during branching morphogenesis. Thinning of BM regions and microperforations becomes visible in areas of actively growing and expanding epithelium in salivary gland, lung, and kidney [7,45]. Previous studies had observed thinning of the BM in lung and kidney by the use of electron microscopy [115,116]. In thinner BM regions,

epithelial and mesenchymal protrusions are observed through the microperforations, allowing for mesenchymal–epithelial contacts [117]. These contacts might permit epithelial–mesenchymal inductive signaling [118] as well as stimulating bud growth and promoting overall expansion of the tissue [119].

The microperforations are present in end buds at the highest frequency during the period of rapid cell proliferation and bud expansion preceding the cleft [7]. Although microperforations are evident around the expanding end buds, they seem to be absent in both the cleft and around the duct, where epithelial expansion is not expected; in fact, there is more accumulation of the BM in these latter two regions (Fig. 13.7) [45,115]. Greater accumulation of BM components is consistent with higher basal lamina integrity at these sites, which is compromised at the distal portion of end buds due to the microperforations to permit expansion.

The BM is remodeled locally by dilatation and contraction of individual microperforations. These constant stretching processes at the microperforations are mediated by actomyosin-dependent physical distortion involving nonmuscle myosin II [45]. In embryonic salivary gland the microperforations are small enough to allow for protrusions from either epithelial or mesenchymal cells without intermixing of cells across the BM. This is not the case in the lung, where cell crossings are observed. BM microperforations in the lung can permit inflammatory cells to enter into the epithelium, suggesting that this mechanism in the lung (and potentially other tissues) may serve as a mechanism for cross-tissue interactions [117].

In addition, however, during branching, the BM is remodeled globally through translocation of the entire BM from the bud tip down to the secondary duct. Direct proteolysis is essential for both BM translocation and generation of microperforations, and protease inhibition prevents both the formation of microperforations and this translocation of intact BM [45]. As the organ grows, BM needs to be remodeled to allow for tissue expansion while still providing structural support and separation of epithelial from mesenchymal compartments. Stromal cells and epithelial cells that migrate during branching likely remodel the BM by releasing proteases and producing components of the BM. Glycosaminoglycans (GAGs) have also been shown to play a role in BM turnover. GAGs are degraded at a higher rate in the end buds during branching compared to the cleft regions, while in the stalk it remains constant [96]. An increase in GAG degradation also correlates with a decrease in basal lamina integrity, so the processes of GAG and protein degradation are likely to be linked.

In addition to GAG degradation in the end buds, salivary gland collagen IV has also been shown to be degraded as the result of proteolysis. Membrane-type matrix metalloproteinase-2 (MT2-MMP), a membrane-intercalated member of a collagenolytic enzyme family, is known to be responsible for collagen IV ECM degradation in embryonic salivary gland. MT2-MMP proteolysis releases NC1 domain fragments as a product of the proteolysis of collagen IV chains, which can further stimulate MT2-MMP and genes associated with proliferation to promote branching through its binding to β1 integrins [34,103].

ECM degradation and cell signaling have been postulated to increase not only distensibility but also cell tension [28] in the end buds. As the epithelium expands, it stretches the BM. The physical forces and perhaps the epithelial membrane protrusions likely contribute to generating the local BM perforations. Therefore degradation of the ECM is important for both global and local

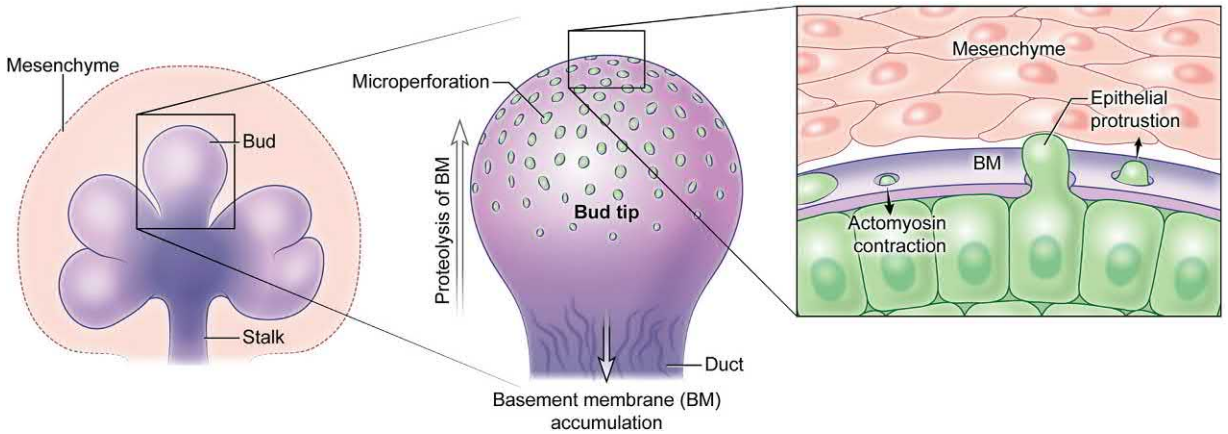

FIGURE 13.7 Basement membrane microperforation. During branching morphogenesis, microscopic holes of varying sizes appear in the basement membrane of expanding buds as a result of protease degradation of the basement membrane and actomyosin contractility. The epithelial cells extend protrusions through holes in their vicinity, and as they push outward, they dilate the perforations in the basement membrane. They eventually retract using actomyosin contractility. Both myosin II–mediated contractility and basement membrane microperforations contribute to the local and global remodeling observed in the salivary gland.

remodeling by influencing cell behavior and providing sites for the epithelial cells to extend protrusions and dilate the BM as they push through microperforations, generating regions of epithelial−mesenchymal contacts (Fig. 13.7). Once the microperforations disappear, BM accumulates to provide support to the gland and allow differentiation to begin. Therefore this phenomenon of global translocation of BM can provide support and help to shape the tissue [45].

Current evidence indicates that BM remodeling is mediated by an increase in protease activity plus myosin II mechanical forces. Myosin II is essential for maintaining the integrity of the microperforations during the translocation of the BM, allowing the cells to reshape and remodel the BM by actively pulling on it through actomyosin contractility [45].

Mathematical and computational models

In order to identify underlying mechanisms of branching morphogenesis, as well as to understand how they differ between different organs, researchers have generated mathematical and computational models that are beginning to provide new insights. These models have been primarily directed toward explaining how signaling mechanisms, geometry, or mechanical forces contribute to branching patterns.

Geometry

Fractal geometry has provided a conceptual basis for identifying mechanisms that may underlie the formation of branches in biological settings. Fractal geometry has been applied to lung branching by considering the lung as a fractal object lacking characteristic scale and having self-similarity [29,120,121]. Some of the modeling for lung geometry uses exponential models, starting with the premise that the lung is a dichotomous branch tree with a parental branch of greater length and diameter and an exponential decrease with every daughter branch due to maximizing entropy. Nevertheless, when this model was used to analyze more than 10 generations of branches, the exponential decrease could no longer accurately predict the diameter and length of subsequent branches. Therefore a renormalization model was developed in which the changes originally considered exponential were shown to be power-law decreases modulated by harmonic variation of the mean of the branch diameter [122].

Mechanical forces

A different type of model that focuses on mechanical forces during early lung branching predicts a linear relationship between epithelium growth and strain rate by the mesenchyme, consistent with the stress−strain rate of fluid flow. In this model the epithelium maintains a tangential stress similar to the surface tension between two fluids, in this case the mesenchyme and the luminal fluid [123]. However, the mesenchyme could be considered as two fluids itself, because there is dense ECM around the epithelium and the outer layer is less dense. There is also evidence suggesting that the branching in the lung is not determined by the ratio of viscosities but instead by residual mechanical stress [124]. Such residual stress could come from cross talk between the mesenchyme and epithelium, important not only for stimulating mesenchymal proliferation and epithelial bud outgrowth but also for governing internal forces. Since the mesenchyme will grow along with the epithelium, this factor, as well as the forces exerted by the boundaries of neighboring organs that compete to fill the available body space, could contribute to pressure gradients determining the overall position of the lung [125].

It appears that the first three generations of branches in the developing lung are stereotyped due to competition to fill the available spaces, but that there is some variability in angle and time of branch generation in later branch generations as the available space is occupied by the organ, accompanied by a relaxation of the stereotyped pattern. These concepts might also help to explain why kidney appears to be stereotyped or deterministic early in development, while the pattern is more stochastic at later developmental stages.

In addition to the mesenchyme, smooth muscle can also contribute to mechanical forces and constraints in developing organs. For example, smooth muscle has been shown to serve as an external physical constraint to epithelial expansion in the developing intestine, resulting in epithelial folding [126,127]. A similar mechanism involving differentiated smooth muscle as a girdle-type restraint around the tip of lung epithelium is thought to play a role in initiating the first cleft in lung. This first cleft is observed at approximately E10.5, but it is not until E11 that nerves are detected around the smooth muscle—this fact is most consistent with simple physical constraint of the expanding epithelium by smooth muscle cells rather than active contraction; the smooth muscle could subsequently become contractile but only after initial branching [33]. Nerve innervation and active smooth muscle contractility could contribute to lung mechanical forces after E11—contributing to fluid flow within the lumen through periodic smooth muscle contraction and relaxation.

Signaling mechanisms

A ligand receptor−based Turing model has been used to explain patterns of signaling and tissue remodeling during lung and kidney branching morphogenesis. This proposed

mechanism involved two factors—a ligand and a receptor—which have different diffusion rates, with their interaction leading to upregulation of receptor levels. The biological variables in this Turing model included the ligand, receptor, inhibitor, and cell differentiation. Application of this model to the lung has been used to explain clefting and stalk elongation. The initial process of bifurcation was modeled to arise as a result of the bud moving toward the activator (ligand). At some point, there could be insufficient receptor (substrate), and a new group of cells without bound ligand might then deviate their motion to fulfill the demand and promote branching. Stalk elongation would be explained as a result of lateral inhibition of new branch formation [128].

Application of the ligand receptor−based Turing mechanism to lung involved FGF10 and sonic hedgehog (SHH), and application of this analysis to GDNF-RET signaling in the kidney was also quite effective. It revealed that the pattern of branching observed in developing wild-type and mutant kidneys could both be predicted by the computational model. In addition, there was positive feedback between GDNF and Wnt11 to enable kidney buds to grow into close apposition to permit closely packed glomeruli [129]. This highly effective model does not yet, however, explain the shapes (length, width, and angle) of the branches, or how other factors, such as mechanics and the other cell types in these organs, work together to achieve efficient branching and the final organ architecture.

Agent-based models, such as cellular automata, consider epithelial cells as individuals instead of collective computational agents, allowing for single-cell studies [130]. Application of these models to GNDF signaling in the kidney suggests that GDNF-mediated proliferation is sufficient for generating branching, since proliferation independent of GDNF produces no branching, that is, GDNF is essential for ureteric bud initiation and overall branching [131]. Ideally, this model could be enhanced by incorporating the cell forces generated by mesenchymal cells that can contribute to branching.

In conclusion, mathematical, physical, and computational models are providing new, intriguing insights into the ways in which mechanical forces and biochemical signaling guide the complex branch structures of tissues. However, they do not yet consider all of the components known to be involved (mechanical, biological, and biochemical signaling) [124]. In order to achieve a better understanding of the process of branching morphogenesis, it will be valuable to integrate biological and biochemical experimentation closely with computational modeling, so that the novel predictions from models can guide experiments, and the biological results can refine the models.

Conclusion

Branching morphogenesis is a topic that has fascinated researchers for many years. This fascination arises from a desire to understand the dynamic changes in embryonic epithelia, signaling, and ECM that lead to complex tissue architecture and efficient function in compact organs. More recently, an understanding of branching morphogenesis has proven to be valuable for potential future regenerative medicine. An example of this involves salivary glands, the function of which is disrupted in Sjögren's syndrome and radiation therapy for head and neck cancer [132]. The effects of radiation therapy have been mimicked in mouse models, with effects on progenitor cells [51], cell proliferation [46], nerve innervation [40], and gland secretory functions [132]. Studies of branching morphogenesis in this gland established that even after dissociation of the gland into single cells, the cells retain the capacity to self-aggregate and regenerate into bud-like structures [52]. The subsequent identification of transcription factors that are involved in self-assembly has led to the development of an engineered salivary gland that upon transplantation into mice with salivary gland defects can restore secretory function of the glands [54]. These results provide promise to patients who suffer from xerostomia and indicate how an understanding of branching organs can help develop technologies and treatments to restore proper organ architecture and function. This and other examples in other organ systems provide hope that a better understanding of branching might eventually lead to engineering of organs that can reduce the waiting time for transplants and increase patient life expectancy for conditions, such as pulmonary diseases and kidney failure.

Advances in genome editing are providing tools that should enable the study of gene function [133] in organ explants and cultures. These and other manipulations should help elucidate biochemical signaling in branching organs. In addition, knowledge gained from understanding cell−cell interactions and cell−matrix interactions during branching can help in the development of biomaterials and microfluidic devices that better mimic the tissue microenvironment.

Advances in omics (including genomics and proteomics) are helping to identify subpopulations of cells important during gland branching [134]. This knowledge is helping researchers better understand which cells are playing important roles during branching in specific glands and verify whether the genes required to establish these cell populations are conserved across branching organs. In addition, advances in computational biology have helped design better predictions by integrating data from sequencing or other big data technologies with experimental data on mechanical, biological, and biochemical signaling to produce more accurate models.

Although these technologies have provided insight into the mechanisms of mechanotransduction, branching patterns, and biochemical signaling during branching, many questions remain. For example: why is a specific ligand–receptor signaling system used primarily in one organ but not in the others? How do tissues coordinate cleft formation with both local proteolysis and global translocation of BM? How do microRNAs regulate different aspects of nerve innervation, cell–cell interaction, and cell–matrix dynamics during branching morphogenesis? What are the advantages and disadvantages of one branching pattern over the other? Does the stereotyped branching pattern of the lung help increase later gas exchange in this organ? Why is kidney branching stereotyped initially, but not in later steps? Even more importantly, what could happen if these developmental mechanisms that are so crucial to proper organ structure and function are aberrantly activated during adulthood?

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Chapter 14

Mechanobiology, tissue development, and tissue engineering

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Introduction

Mechanobiology is a field of biology that investigates the generation and response to mechanical signals by cells or tissues. In recent years, our understanding of mechanobiology has expanded to include not only the multitude of cellular responses to mechanical signals, but also how cells generate mechanical forces to communicate with their environment and neighbors. At the intersection of biology, physics, and engineering, mechanobiology has contributed greatly to both our understanding of biological phenomena such as morphogenesis, tissue regeneration, and cancer metastasis, and the development of medical applications such as implants, wound regeneration control, and tissue engineering.

This chapter covers the basics of mechanobiology with a focus on the human body, beginning with an introduction of the existence and generation of mechanical signals within biological systems. The discussion then focuses on the ability of cells to detect and respond to these signals, termed mechanosensing and mechanotransduction, respectively, followed by the underlying mechanisms that lead to responses in higher order structures such as tissues and organs. The chapter concludes with examples of how mechanobiology-derived design elements have been implemented in tissue and organ engineering.

Mechanical forces in biological systems

All biological systems receive mechanical signals from a range of internal and external sources (Fig. 14.1). Some of these sources generate localized forces confined to the immediate environment of a cell, while other sources generate macroscale forces that affect an entire tissue. A typical tissue comprises many distinct populations of cells, where mechanical forces may affect these populations similarly or differently, depending on tissue architecture and other compositional differences. The initiation, sustainment, and release of macroscale forces may each affect tissues and trigger coordinated responses. In addition, cells and tissue also use mechanical forces to sense the mechanical environment such as stiffness. In mechanobiology, the main forces of interest are tension, compression, and fluid shear.

Tension

Tension is a force met by the resistance of a material against an increase in its length. Some sources of tension are external, such as from physical tugging of the skin. Others arise within the body itself, exerted by tissues, such as muscle, upon themselves and/or other tissues.

Skin, organ linings, and other similar tissues comprise flat sheets of cells and are thus referred to as epithelial tissue. Epithelial tissues experience tension both constitutively from cell-generated forces and externally from external sources. One example of epithelial tension is the cyclical tension exerted upon the lining of the lungs during breathing [2]. This cyclical tension is necessary for the proper development and maintenance of the lung epithelium, as improper tension can cause defects such as fibrosis and emphysema of the lungs [3–5].

The human body also generates tension for its maintenance and movement. Skeletal muscles pull on tendons, other muscles, and skeletal components both constitutively and during voluntary movement. As a result, tendons, thick cords of connective tissue that connect skeletal musculature to the skeleton, are under constitutive tension. These tensile forces cause strengthening and remodeling responses in connective and other affected tissues [6–8].

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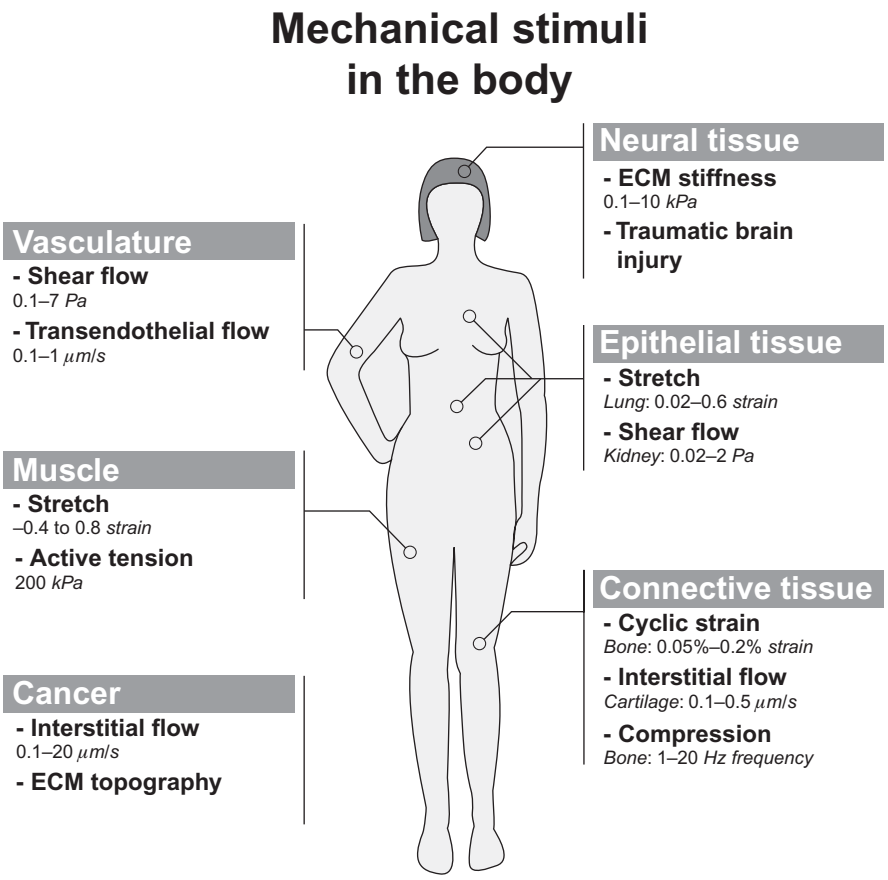

FIGURE 14.1 Mechanical stimuli found in various tissues inside the body. The main forces found in tissues are tension, compression, and fluid shear, which may allow cells and tissues to sense strain, stiffness, or other parameters responsive to mechanical forces. Excessive forces may cause damages such as traumatic brain injury from rapid compression. *Adapted from Polacheck WJ, Li R, Uzel SGM, Kamm RD. Microfluidic platforms for mechanobiology. Lab Chip 2013;13(12):2252–67 [1]. Published by the Royal Society of Chemistry.*

Compression

Compression, forces met by the resistance of a material against a decrease in its length, is exerted both as a constant, such as in the case of gravity while standing still, and variably as a result of motion [7]. Compression affects both soft tissue and bones. The repetitive compression–decompression cycles of walking exert variable mechanical stimuli superimposed on the constant forces of standing. Bones change their shape, density, and stiffness in response to changes in the conditions of mechanical loading, such as during space flights [9]. Improper loading conditions also cause osteoporosis, osteoarthritis, and fibrosis, while proper exercises assist in maintaining bone structure [9]. Compression also causes changes in interstitial fluid flow and contributes to fluid shear forces [7,9].

Fluid shear

Fluid shear is a drag force oriented parallel to the surface caused by the flow of fluid past a material. Both cardiac and smooth muscles operate by contracting around a fluid to direct flow, which causes heterogeneous patterns of pressure and shear stress to the vasculature [10]. Normal levels of blood pressure and shear flow help maintain the physiological condition of vasculature, while shear flow in the gastrointestinal tracks has been implicated in the release of serotonin and regulation of intestinal fluid secretion [11]. Conversely, abnormal fluid shear is believed to directly or indirectly cause atherosclerosis, cardiac fibrosis, and other systemic effects [12,13].

Both hearing and proprioception are driven by fluid flow against the inner surface of the cochlea. Hearing initiates with vibrations of the eardrum, which are transduced into patterns of fluid waves inside channels within the cochlea [14]. Head tilt is also transduced into fluid flow within three semicircular canals in a separate part of the inner ear, known as the vestibular system [15]. Mechanosensitive hair cells on the inner surface of these channels sense the fluid flow in both cases [14–16].

Shear force also occurs from interstitial fluid flow through solid tissues. In the brain, a link has been found between increased cerebrospinal fluid flow and the invasion of glioblastoma [17]. Similar findings have been found for other cancer cell types, suggesting that interstitial flow may play a general role in metastasis [18].

Cellular mechanosensing

The mechanotransduction of larger tissues is often dependent on the ability of their constituent cells to sense and

respond to microscale forces, termed cellular mechanosensing. Individual cells experience microscale mechanical stimulations as macroscale forces travel through the heterogeneous and complex tissue architecture. These microscale forces are characterized by a magnitude in the order of piconewtons and a spatial resolution in the order of microns. Even in the absence of macroscale forces, cells continuously exert microscale forces on the surrounding environment while sensing the feedback responses in a complex feedback loop. The combined architecture of the cytoskeleton, junctions, and microenvironments forms a complex, dynamic framework that allows cells to sense and respond to forces.

The cytoskeleton

All mammalian cells are enclosed in a cell membrane. Inside the cell, nucleus and organelles are suspended in a viscoelastic cytoplasm that allows for the intracellular transfer of forces. The generation, transmission, and balance of these forces are mediated by a dynamic proteinaceous network called the cytoskeleton, comprising three types of filaments known as actin filaments, microtubules, and intermediate filaments. These filaments, formed by noncovalent associations of monomeric subunits, are capable of spanning large distances within the cell.

Actin filaments, the predominant structure that transmits forces across the cell, are polarized, semiflexible chains. Different isoforms of the motor protein myosin may pull actin filaments along defined but different directions relative to filament polarity [19]. Actin, myosin, and accessory proteins, such as cross-linking factors, form a contractile network that both generates and sustains tension within the cell [20]. In addition, contractile bundles of actin and myosin, known as stress fibers, are prominent contractile structures often viewed as the counterpart of myofibrils in muscle cells [21,22]. Stress fibers terminate at one or both ends at focal adhesions, which are discrete transmembrane plaques that anchor cells to the substrate and allow the transmission of forces from stress fibers to the substrate for generating traction forces [23]. While stress fibers are most prominent in stationary cells, some migrating cells assemble transverse arcs of actin and myosin bundles behind the advancing edge without direct association with focal adhesions [23]. The contraction of these arcs serves to pull itself away from the curved advancing edge in a process called retrograde flow, which exerts tension through connections with other intracellular structures for driving directional transport during cell migration.

Microtubules are structural fibers composed of linear protofilaments associated laterally for forming long hollow cylinders. They are commonly perceived as rigid supports that are able to withstand compressive forces such as those generated by the actomyosin network. In addition, microtubules guide intracellular transport, where they provide a path for motor proteins, such as kinesins and dyneins, during the transport of vesicles [24]. Similarly to actin filaments, microtubules are dynamic and polarized structures, showing different rates of polymerization and depolymerization at their two ends. By binding to microtubules and affecting these rates, microtubule-associated proteins can modulate the organization of microtubules [25]. Regulation of the microtubule network affects the transport of organelles, signaling factors, and other important proteins for generating far-reaching effects, including polarization, migration, and proliferation [26]. In addition, microtubules interact with the actin cytoskeleton at focal adhesions and possibly other structures, where they may regulate the assembly and disassembly of actin structures [27].

Intermediate filaments are a family of related, cell-type dependent filaments that are laterally assembled from unit length filaments composed of octamers of tetramers [28]. Vimentin is largely expressed in mesenchymal cells, while cytokeratin is largely expressed in epithelial cells. Both interact with actin filaments and microtubules, desmosomes, and hemidesmosomes [29]. Intermediate filaments have been hypothesized to passively resist cellular deformation. Under tension, intermediate filaments may reorganize their protein conformation to increase their resistance to strain [30,31]. Individual cells have been found to respond differently to shear flow in vitro in the presence or absence of vimentin [32], and deficiencies in intermediate filaments are known to cause skin-blistering diseases [33,34]. A distinct class of intermediate filaments called lamins are expressed in most eukaryotic cells to form a shell around the nucleus, for the purpose of resisting nuclear deformation and transmitting mechanical signals between the nucleus and the surrounding cytoplasm [35]. Lamins are mechanically tethered to the nucleus through a mechanosensitive complex known as the linker of nucleoskeleton and cytoskeleton (LINC) complex, which also associates with actin filaments and microtubules [36] to possibly mediate the mechanical crosstalk.

Stretch-activated ion channels

Stretch-activated ion channels respond to membrane deformation and play critical roles in sensing vibration, pressure, and touch. A stretch-activated ion channel is composed of transmembrane proteins encapsulating a hollow channel that opens upon tension. Ions flow rapidly through an open ion channel, causing immediate changes in transmembrane potential and intracellular calcium concentration, which allow for rapid signaling responses in the order of milliseconds [37]. These channels are present

in the cochlea, smooth muscle, hair follicle cells, and cardiac tissue [37]. Examples of mammalian stretch-activated ion channels include the transient receptor potential family of channels and the recently discovered piezo channels [38,39]. These stretch-activated ion channels have been shown to mediate the sensing of fluid shear in cochlea hair cells and in small intestine epithelial cells during the development of microvilli [11,14–16].

Cell–cell adhesions

Cell–cell adhesions consist of discrete junction structures responsible for mechanically coupling cytoskeletons together and allowing the transmission of mechanical signals across a cell collective [40]. The majority of these anchoring junctions are found in epithelial and endothelial cells, although some are also observed in mesenchymal or cancerous cells [41].

Adherens junctions form transcellular mechanical linkages between the actin filament networks. Their primary component is a transmembrane protein family called cadherins, which generally bind to cadherins of the same type in neighboring cells. Different classical cadherin subtypes, such as E-cadherin, N-cadherin, and VE-cadherin, are expressed in different tissues and at different times in development [41,42]. The homophilic binding of these cadherins is a driving factor for cellular sorting and morphogenesis [41]. Upon intercellular binding and tension, cadherins undergo a conformational change that alters the binding properties of its cytoplasmic domains to cytoplasmic proteins, such as catenins, initiating signaling cascades [43]. β-Catenin is stabilized at the adherens junction under tension, which in turn inhibits nuclear relocation and transcription of several signaling factors in the Wnt signaling pathway [43]. At least some of the associated mechanotransduction processes involve changes in protein conformation. For example, α-catenin, which mediates the binding of β-catenin to actin, unfolds under force to expose binding sites for other actin-related proteins such as vinculin and α-actinin (Fig. 14.2) [44]. Interestingly, many of these proteins also associate with focal adhesions, suggesting that there may be crosstalk and shared mechanisms between cell–cell adherens junctions and cell–substrate focal adhesions [45].

Desmosomes form another type of mechanical linkage between cells through the associated cytokeratin networks [47]. Their primary components are transmembrane proteins called desmoglein and desmocollin, which bind to each other and to other desmogleins and desmocollins on the surface of adjacent cells. Their cytoplasmic domains bind to several accessory proteins such as desmoplakin, plakoglobin, plakophilin, and several catenins that are also associated with adherens junctions [47]. Desmosomes are predominantly found in tissues that experience intense mechanical stress, such as cardiac muscle, bladder, and epithelia. Recent studies suggest that desmosomes typically experience low levels of tension under normal conditions but are subjected to and withstand high tension under external loading [48,49]. As intermediate filaments do not generate contractile forces, desmosomes appear to be passive mechanical adhesions that protect the tissue against external forces [49].

Cell–substrate adhesions

In addition to contact with other cells, adhesive cells mechanically tether themselves to the substrate via cell–substrate adhesions. The substrate typically consists of secreted proteins called the extracellular matrix (ECM). Like cell–cell adhesions, the cytoplasmic side of these adhesions is also associated with the cytoskeleton and signaling proteins, which allows cell–substrate adhesions to respond to mechanical forces by affecting a wide variety of cellular pathways. The best characterized cell–substrate adhesions are focal adhesions and hemidesmosomes.

As described earlier, focal adhesions are transmembrane structures associated with the actin cytoskeleton and signaling proteins on the cytoplasmic side. The exterior side of focal adhesions binds to ECM proteins, such as fibronectin and collagen, via a family of transmembrane proteins called integrins [50]. Integrins are heterodimers of alpha and beta subunits. Different combinations of alpha and beta subunits form different heterodimeric complexes to allow the recognition of different extracellular ligands. Binding of integrins to ECM ligands triggers a conformational change that is sensed by intracellular focal adhesion proteins [51]. Focal adhesion proteins, including focal adhesion kinase, paxillin, talin, vinculin, zyxin, vasodilator-stimulated phosphoprotein, and α-actinin, interact with each other to form a complex network of signaling pathways for transducing mechanical signals [52,53]. Some of these proteins are known to respond directly to forces by changing the conformation and initiating signaling cascades. For example, stretching of talin and vinculin causes a conformational change, which then exposes additional binding sites for downstream signaling proteins (Fig. 14.3) [54–56]. Mechanical stimulus through focal adhesions also induces the phosphorylation of tyrosine residues on some of these proteins, which affects their interactions with other proteins [57,58]. Such events then lead to several key cellular responses, including adhesion, proliferation, migration, and differentiation.

Hemidesmosomes are predominantly found in epithelial cells, acting as linkages between the ECM and intermediate filaments such as cytokeratin. They are primarily composed of a specific integrin, α6β4, and plectin 1a,

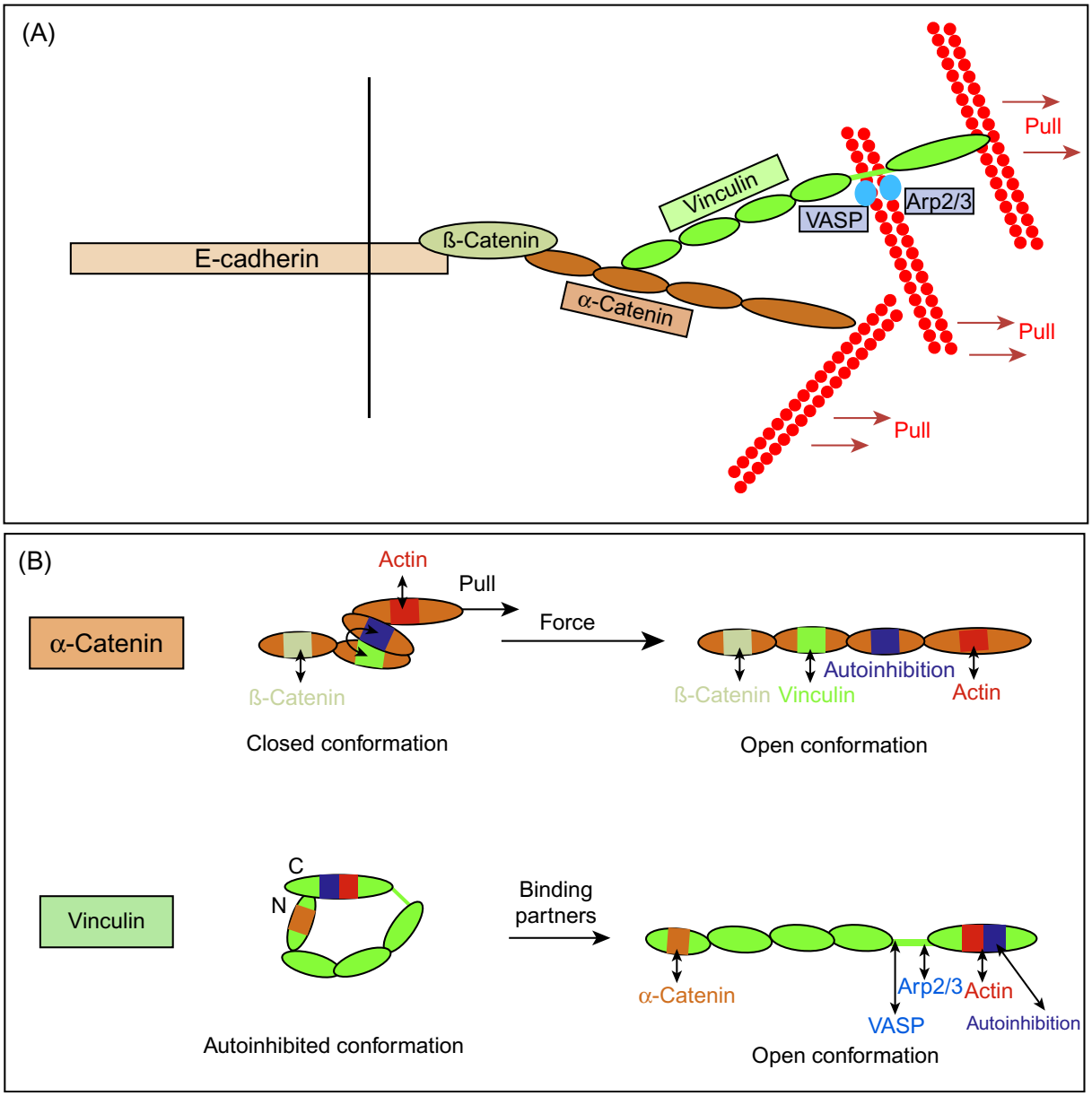

FIGURE 14.2 **Tension sensing at adherens junctions.** At nascent adherens junctions, E-cadherin interacts with two tension-sensing proteins: α-catenin and vinculin. Upon homodimerization with E-cadherin on opposing membranes, E-cadherin binds α-catenin on its intracellular domain and becomes linked to the contractile actomyosin network (A). In response to either external force or actomyosin contractility, α-catenin changes conformation and exposes a cryptic binding site to vinculin. Vinculin itself is also mechanosensitive. While typically existing in an autoinhibited state, it unfolds upon the binding of α-catenin to expose binding sites for downstream partners and to promote the maturation of adherens junctions (B). *Adapted from Roper K. Integration of cell–cell adhesion and contractile actomyosin activity during morphogenesis. In: Yap AS, editor. Current topics in developmental biology. Amsterdam: Elsevier; 2015 [46].*

which are both transmembrane proteins, along with their associated cytoplasmic proteins. Recent studies indicate that tension exerted by muscles or external pressure can induce hemidesmosomes to mature from punctate spots to larger stripes and activate intermediate filament phosphorylation in *Caenorhabditis elegans*, raising the possibility that vertebrate hemidesmosomes may also exhibit sensitivity to forces [60].

The extracellular matrix

The ECM is the complex extracellular scaffold of proteins that cells deposit and adhere to. Cell adhesion to the ECM is involved in the control of cell spreading, migration, and proliferation [61,62]. ECM is present in all solid tissues from bone to fat, showing a full range of stiffness. It serves several major roles at the tissue level: presenting

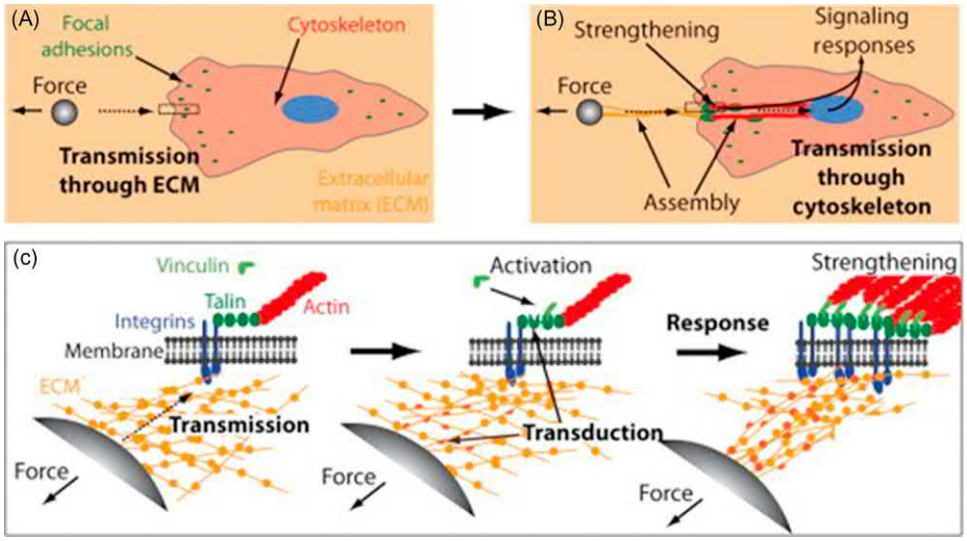

FIGURE 14.3 **Tension sensing at focal adhesions.** Mechanical properties, such as external tension, stiffness, or internal contractility, are transmitted through the extracellular matrix and sensed as tugging forces at nascent focal adhesions (A). These forces generate signals within the cell (B) that promotes the maturation of focal adhesions and strengthening of cell−substrate adhesions. At the molecular level, forces on nascent focal adhesions cause talin to undergo a conformational change (C) and expose cryptic binding sites for vinculin. Vinculin also contains cryptic binding sites for other focal adhesion−associated proteins that are exposed upon binding to talin. Other force-mediated pathways also contribute to integrin clustering and strengthening of mechanical connections with the contractile actomyosin network. *Adapted from Hoffman BD. The detection and role of molecular tension in focal adhesion dynamics. In: Engler AJ, Kumar S, editors. Progress in molecular biology and translational science. Amsterdam: Elsevier; 2014 [59].*

sites for cell anchorage and migration, protecting cells from direct impact of macroscale forces, converting and transmitting macroscale forces as microscale forces for cell sensing, providing mechanical signals such as stiffness, and serving as a reservoir of signaling molecules and enzymes [61,63]. A large body of evidence supports the role of ECM as an active player and therapeutic target in diseases such as fibrosis, atherosclerosis, aging, and cancer [63,64].

There are two major forms of organization for ECM. Connective tissue comprises fibrous three-dimensional (3D) meshes of fibrillar collagens, proteoglycans, and glycosaminoglycans [65]. Mesenchymal cells attach to and migrate along these fibers. Fibrillar collagens are the main structural component of ECM in connective tissues. The most abundant fibrillar collagen is collagen I, which forms thick, highly ordered fibers that are resistant to bending. Connective tissue also contains elastin, a loose, poorly organized protein that provides elasticity [65].

The second form is the basement membrane, which are sheet-like substrates that provide a two-dimensional (2D) surface for the attachment of epithelial and endothelial cells [66]. Basement membranes are composed primarily of collagen IV and laminin. Unlike collagen I, collagen IV forms thin meshworks instead of tight fibers, while laminin is a glycoprotein that self-associates to form sheets [67]. Both basement membrane and connective tissue contain proteoglycans and glycosaminoglycans, which are hydrophilic macromolecules that facilitate water retention. Additional proteins in ECM, such as fibronectin, thrombospondin-1, and tenascins, do not influence bulk mechanical properties of the tissue but nevertheless play important roles in modulating cell−ECM interactions and nanoscale mechanical signals [65,67].

ECM is considered a viscoelastic material, as it has both viscous and elastic properties [68]. The elastic properties of ECM are largely attributed to collagens and elastin, while the viscous properties of ECM are attributed to both the slippage of collagen fibers against each other and the restricted flow of fluid through proteoglycan and glycosaminoglycan networks [69]. Many forms of ECM contain a substantial amount of fluid, either in pockets or dispersed throughout the matrix. For example, the matrix in bone marrow contains interstitial fluid within channels termed lacunae and canaliculae, while cartilage contains fluid evenly distributed throughout the matrix [9,70]. Macroscale forces of tension and compression cause the ECM to exert differential pressures on these fluids, converting these forces into shear flow sensed directly or indirectly by cells. Through its structure and composition, ECM serves as a passive transducer of macroscale forces into mechanical signals.

The ECM also acts as a direct mechanotransducer through the exposure of cryptic binding sites and associated growth factors. Forces induce partial unfolding and

fibrillogenesis of ECM proteins, such as fibronectin, causing changes in cell−ECM adhesion and downstream responses [71]. In addition, forces promote the release of soluble factors, such as TGF-β and VEGF, that can regulate cell growth, differentiation, and ECM remodeling [72−74].

Cellular effects of mechanotransduction

Mechanotransduction is the conversion of external mechanical signals into intracellular chemical or physical events, which are directly responsible for the responses of tissues to bulk forces and other physical stimuli. Many fundamental behaviors of cells are affected by mechanotransduction, such as adhesion, spreading, migration, proliferation, differentiation, and cell−cell interactions within collectives [75].

Substrate adhesion, spreading, and migration

Many types of cells adhere dynamically to substrates through focal adhesions. Initially punctate in morphology, nascent focal adhesions are pulled by forces from the associated actin filaments toward the center of the cell against the resistance of the external substrate, generating tension that causes the focal adhesion to mature and grow larger [76,77]. Conversely, focal adhesions in the rear of migrating cells are weakened by the forces of forward migration generated elsewhere in the cell [50]. Cells are known to form larger and more mature adhesions on stiffer substrates due to increased tension. These adhesions are also more stable and show less turnover [50].

Upon adhesion to stiff substrate surfaces, many types of cells spread out by forming protrusions called lamellipodia and filopodia. Cells on a stiffer substrate are generally flatter and more spread out [78]. The application of cyclic stretching forces further causes cells to spread preferentially parallel or perpendicular to the direction of stretching, depending on the cell type and the rate and magnitude of stretch [79,80]. Cell spreading is regulated by the Rho family small GTPases Rho, Rac, and Cdc42. The activation of Rac and Cdc42 are associated with actin polymerization at the leading edge of lamellipodia, while Cdc42 activation is associated with the formation of filopodia [81]. In addition to GTP binding, the activation of both Rac and Cdc42 involves binding to the plasma membrane, which may involve microtubule-mediated transport in a mechanoresponsive manner [82].

Rho family GTPases also play a major role in cell migration (Fig. 14.4). Frontal protrusion during cell migration involves localized activation of Rho, Rac, and Cdc42 at the front of the cell. In addition, RhoA may play a role in the contraction leading to rear retraction (Fig. 14.5) [85]. The localization of Rho family GTPases is mediated by microtubules, which are asymmetrically distributed in migrating cells [82,86]. This notion is supported by the observation that pharmacologically-induced disassembly of microtubules disrupts cell polarity and directional migration [87−89].

Cells respond to not only external forces but also counter forces in response to the forces that they exert on the environment. The latter mechanism allows the cell to sense stiffness. Stiff substrates tend to enhance frontal protrusion [91−93], causing cells to migrate preferentially away from soft surfaces toward stiff surfaces. This phenomenon, referred to as durotaxis [91], is proposed to play an important role in liver fibrosis and cancer metastasis [94−96]. Recent studies show that cells tug on filopodia to sense the stiffness of the substrate in front of them before committing to protrusion [93]. Cells also migrate toward regions of less confinement in order to adopt a preferential, flatter morphology [97]. This response causes cells to avoid one-dimensional lines in favor of 2D surfaces. Inhibition of actomyosin contractility ablates the response, which supports the notion that contractile forces serve as a mechanism for probing the surrounding mechanical environment.

Cell−cell interactions in collectives

Cells often gather in groups in a multicellular organism, where they are mechanically connected to each other through cell−cell junctions. This allows mechanical responses of individual cells to be communicated to others in the collective, generating collective responses. Cell collectives exhibit diverse responses influenced by mechanical interactions between cells [98,99]. For instance, cells tend to migrate toward each other on softer substrates and separate from each other on stiffer substrates [100]. Cell collectives also respond to shallower gradients of stiffness than individual cells [101], possibly as a result of integration of responses from individual cells and/or positive feedback from cell−cell interactions. These responses then lead to the formation of higher order structures such as tissues. In some cases, such as during wound closure of embryos or epithelial monolayers, the process involves the formation of prominent contractile actomyosin bundles along the multicellular circumference of the wound [102−104]; the contraction of the bundles causes the wound to close.

Many of the responses of collectives involve coordinated movements, where the complex behavior is only partially understood. The well-known phenomenon of contact inhibition of locomotion, where cells polarize away from each other upon contact [105,106], likely represents only a small subset of responses to cell−cell interactions. In order to explain phenomena, such as collective migration, this response must be complemented by

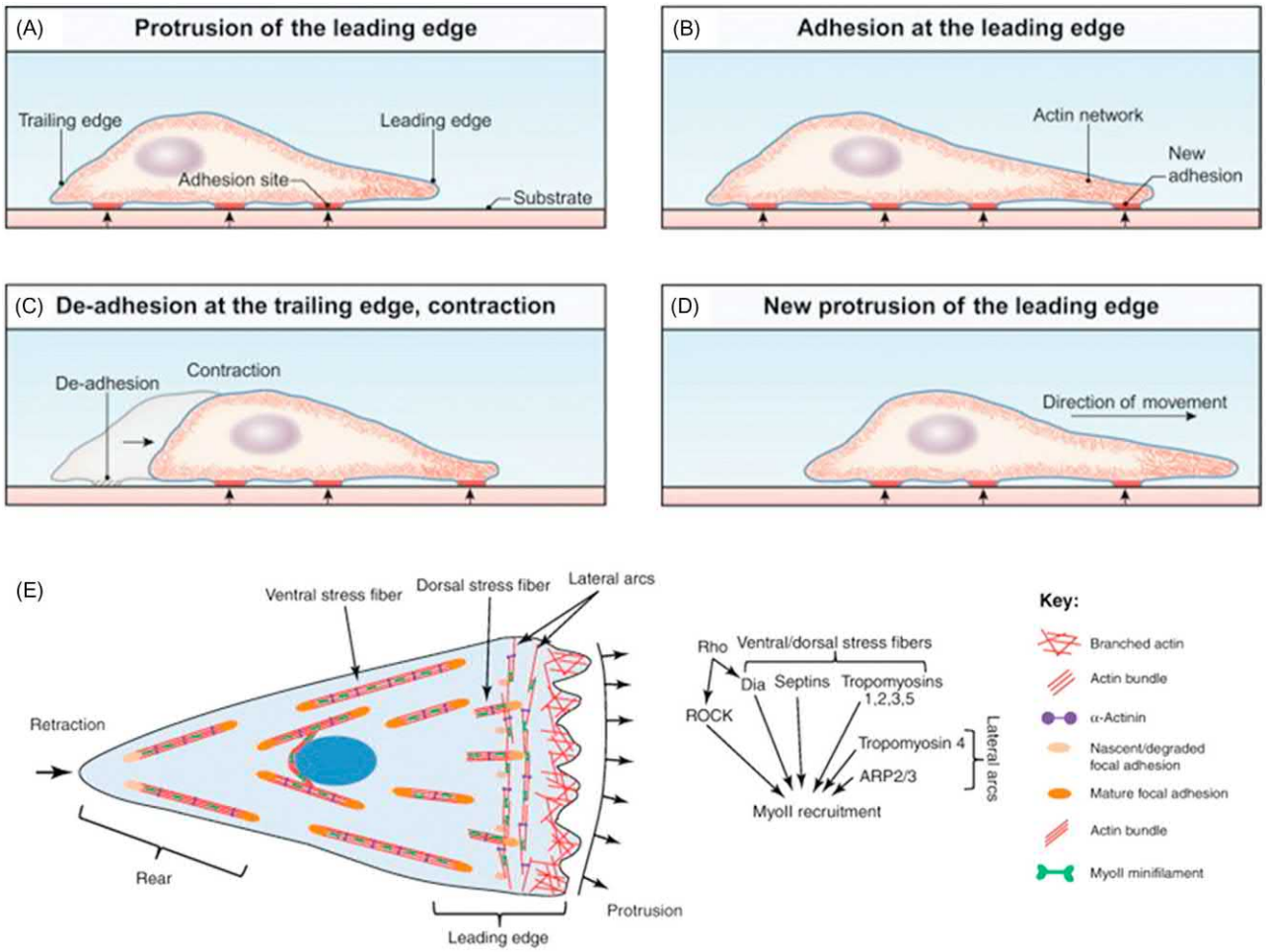

FIGURE 14.4 Mechanical processes involved in cell migration. Cells cycle through distinct processes during cell migration: protrusion of the leading edge (A), substrate adhesion at the leading edge (B), and contraction and release of adhesions from the trailing edge (C). These steps, including new protrusions from the leading edge (D), can occur concurrently, each driven by distinct components of the contractile actomyosin network (E). Actin is organized by myosin II, α-actinin, Arp2/3, and other actin-associated proteins to form the lamellipodia, filopodia, lateral arcs, and stress fibers. Several signaling pathways direct the recruitment and activation of the myosin II motor protein along the actin network to drive contraction and migration. *Adapted from Joo EE, Yamada KM. Cell adhesion and movement. In: Vishwakarma A, Sharpe P, Shi S, Ramalingam M, editors. Stem cell biology and tissue engineering in dental sciences. Amsterdam: Elsevier; 2014; Levayer R, Lecuit T. Biomechanical regulation of contractility: spatial control and dynamics. Trends Cell Biol 2012;22(2):61–81 [83,84].*

other responses. As originally proposed for the slime mold *Dictyostelium* [107,108] and recently observed with cultured mammalian epithelial cells [109], contact following of locomotion allows cells to follow the retreating edge of migrating cells upon head–tail contact. The complementary responses of contact inhibition and contact following may involve opposite mechanical signals upon the contact of different regions of the cell [105,110].

Proliferation and differentiation

Cell proliferation is also affected by mechanical signals. Matrix stiffness, cyclic stretch, and shear flow have all been found to promote proliferation and reduce apoptosis in a number of cell types [111,112]. Proliferation and apoptosis are jointly regulated by multiple mechanoresponsive pathways, including the MAPK pathway, the Hippo signaling pathway, and the MRTF–SRF pathway [112,113]. Defects in any of these pathways may compromise the control of cell growth and tumorigenesis.

MAPKs are activated by increasing substrate stiffness, cyclic stretch, or shear flow. Its downstream signaling cascade causes the relocation of transcription factors to the nucleus [114,115]. The Hippo signaling pathway involves the transcriptional coactivators YAP1 and TAZ, which relocate to the nucleus under increasing external force [116–119]. The MRTF–SRF pathway, with the involvement of myocardin, MRTF-A, and MRTF-B, is affected by mechanical signals through their effects on actin polymerization [120,121]. MRTF-A binds directly

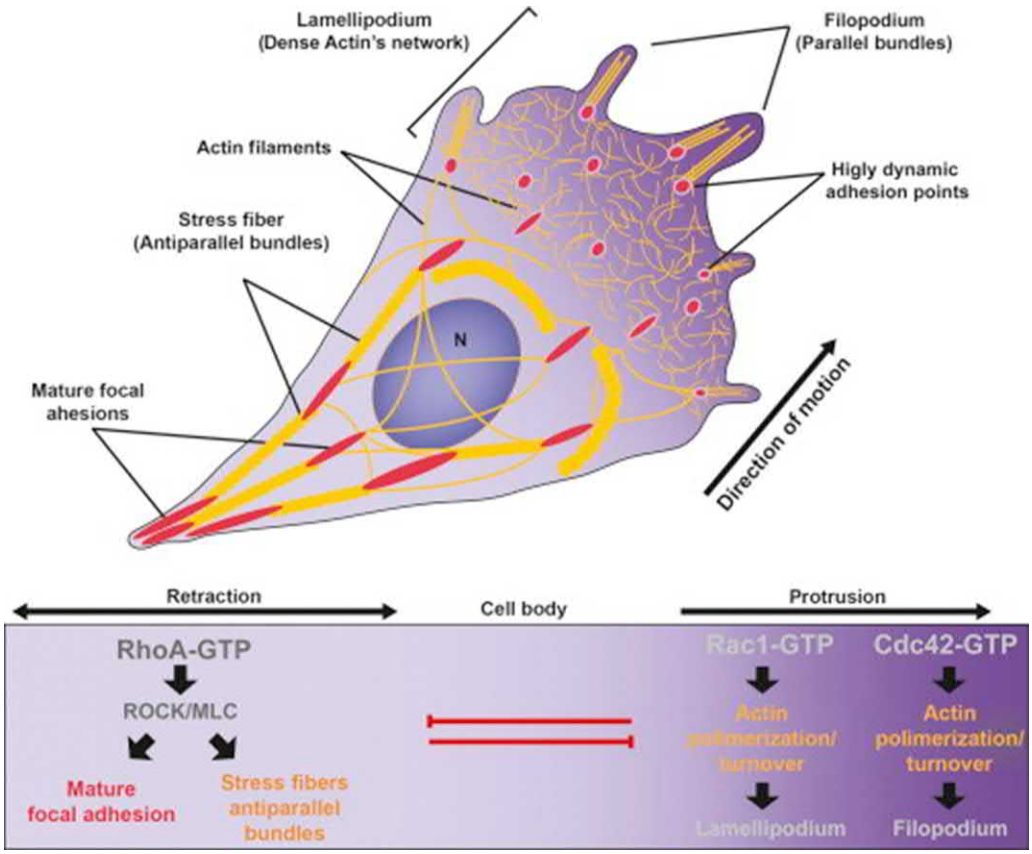

FIGURE 14.5 **Rho family GTPases contributing to cell polarity and actin reorganization.** A possibly oversimplified model of cell migration proposes distinct localization of different GTPases in different regions of a migratory cell to orchestrate the processes of protrusion and retraction. Rac and Cdc42 are found to be active in the leading edge, where they organize protrusions via lamellipodia and filopodia, respectively, while RhoA activities may promote rear contraction and release of mature focal adhesions. *Adapted from Barriga EH, Mayor R. Embryonic cell–cell adhesion: a key player in collective neural crest migration. Curr Top Dev Biol 2015;112:301–23 [90].*

to the actin cytoskeleton to regulate its assembly dynamics [122]. Internal or external stress on the actin cytoskeleton promotes actin polymerization and releases MRTF-A, which then relocates to the nucleus [75]. In all three pathways, the eventual effectors are transcriptional factors, which act to regulate gene expression [123].

Mechanical signals also affect differentiation. Softer substrates, mimicking that of the embryo, promote proliferation of various types of cells [124] as well as morphogenic activities such as neuronal branching [125]. In addition, mesenchymal stem cells differentiate toward neurogenesis, adipogenesis, myogenesis, or osteogenesis in a stiffness-dependent manner [125–127]. Cyclic stretching further guides the differentiation of muscle and tendon cells, while shear flow guides the differentiation of endothelial cells in blood vessels [128–130]. Force-mediated differentiation appears to be regulated by the same pathways that regulate proliferation and apoptosis [75], as both the MAPK and Hippo pathways play a role in the stiffness-sensitive differentiation of stem cells, while MRTF–SRF pathway plays an important role in the differentiation of fibroblasts to myofibroblasts [118,131,132].

Mechanotransduction in biological phenomena

The multitude of mechanotransduction pathways comprises a complex bidirectional signaling network that allows cells and tissues to respond and contribute to the mechanical makeup of the surrounding environment for generating a wide range of biological phenomena (Fig. 14.6). These include physiological phenomena such as wound healing and tissue morphogenesis, as well as pathological phenomena such as cancer metastasis. The examples described in this section serve to underscore the importance of mechanotransduction in biomedical science, engineering, and clinical medicine.

Wound healing

Wound healing involves coordinated migration of multiple cell populations in a manner highly responsive to mechanical cues [129]. After the initial inflammatory and coagulation responses, wounds are sealed by a soft provisional ECM composed primarily of fibrin (Fig. 14.7)

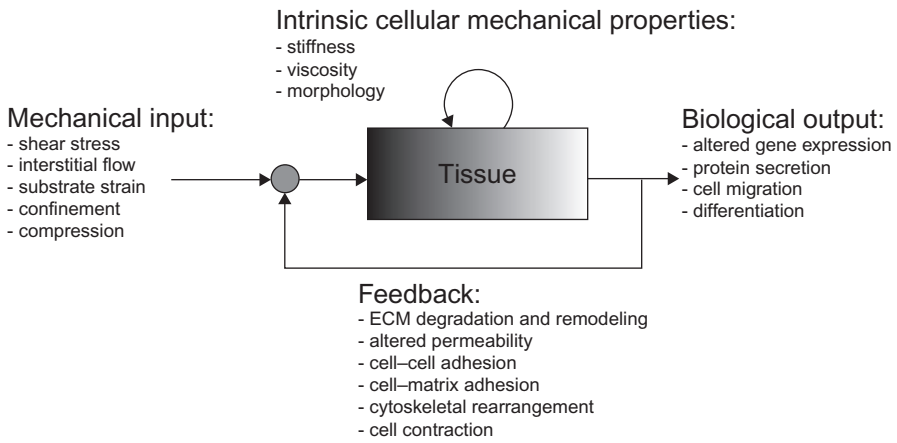

FIGURE 14.6 Conversion of mechanical input into biological output through mechanotransduction. Tissues respond to mechanical stimuli by changing both intrinsic mechanical properties and biological output. Some of these outputs affect the mechanical properties of the cell or tissue, which may constitute a feedback loop to modulate the response. This allows cells to respond to a combination of mechanical signals from both the external environment and themselves. *Adapted from Polacheck WJ, Li R, Uzel SGM, Kamm RD. Microfluidic platforms for mechanobiology. Lab Chip 2013;13(12):2252–67. Published by the Royal Society of Chemistry.*

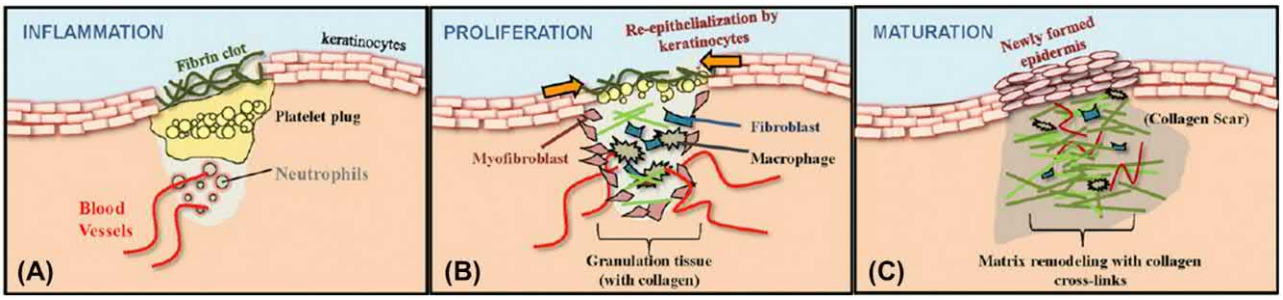

FIGURE 14.7 Progression and mechanics of adult skin wound healing. Shortly after wounding, the injury is covered by a soft provisional matrix consisting of fibrin (A). This is followed by the transformation of fibroblasts into myofibroblasts, which triggers the replacement of provisional matrix with secreted collagen (B), eventually forming stiff scar tissues (C). Simultaneously, the epidermis must migrate collectively to cover the wound (B) and prevent further infection through the compromised tissue. *Adapted from Nauta A, Larson B, Longaker MT, Lorenz HP. Scarless wound healing: from experimental target to clinical reality. In: Atala A, Lanza R, Thomson JA, Nerem R, editors. Principles of regenerative medicine. Amsterdam: Elsevier; 2011 [133].*

[133,134]. ECM stiffness and external tension are sensed by fibroblasts as tension across their focal adhesions [78], which induces their differentiation into myofibroblasts accompanied by the synthesis of collagen, alpha-smooth muscle actin, and profibrotic cytokines to effect permanent scar formation [135,136]. The deposition of collagen by myofibroblasts on the extracellular space further stiffens the matrix, driving a positive feedback loop between ECM stiffening and fibroblast transformation [137]. The mechanosensitive YAP/TAZ pathway is implicated in this pathway, as knockdown of their expression significantly inhibits wound healing [138]. In addition, myofibroblast contraction promotes further transformation of nearby fibroblasts through both integrin signaling and tension-mediated release of TGF-β1 from latent ECM reservoirs [139].

While some scar tissue is generally necessary to maintain tissue integrity, uncontrolled scar formation under pathological conditions may cause fibrosis, which can be cosmetically undesirable and, in the case of surgical adhesions, life-threatening [140]. Specifically, scars formed under excessive external tension have higher cellularity, vascularity, inflammation, and myofibroblast transformation [137], which may compound fibrosis through feedback loops between mechanical cues and matrix stiffening.

During wound healing, keratinocytes must also collectively migrate to cover the surface of the tissue [141,142]. These keratinocytes behave similarly to cell collectives in vitro, showing many of the same responses to mechanical cues. For instance, keratinocytes migrate more collectively and with greater speed and persistence across stiffer substrates [143]. Speed is essential for wound healing to limit infection. It is also beneficial for keratinocytes to migrate collectively because uncoordinated collectives leave gaps in between cells and compromise the integrity of the epithelium.

Thus tension and stiffness have both positive and negative effects on wound healing. While wounds close faster under tension, the risk of hypertrophic scarring is increased. Understanding the mechanical influences on closure speed and scar formation will assist in the identification of optimal conditions for accelerated healing with minimal scarification.

Tissue morphogenesis

Tissue morphogenesis is the process of organizing the spatial distribution of cells during embryonic development. During this process, cells undergo proliferation, migration, and differentiation to form complex tissues and organs. These processes are governed by both soluble factors and mechanical forces. Understanding how these conditions influence morphogenesis will assist not only in understanding many normal and defective developmental processes but also in recapitulating the regenerative mechanism for tissue engineering applications [144].

Embryos are subject to a complex system of forces that guide their development. Mechanical forces and responses play complementary roles in tissue morphogenesis. First, cells can generate forces to induce tissue-wide movements. Second, forces from both inside and outside of the tissue may serve as signals for cells to differentiate and migrate, and for nascent tissues to remodel into specialized organs. These cells may in turn generate mechanical signals or modify their mechanical environment to influence surrounding cells.

Cell-generated forces can lead to complex temporal and spatial patterns of mechanical contraction that orchestrate large-scale movements (Fig. 14.8) [144]. In epithelial cells, these forces are transmitted to neighboring cells through cell−cell adhesions located apically. When the tension across these adhesions is higher than the bending resistance of the supporting substrate, epithelial cells can cause the substrate to lift up and curl in the apical direction in a process termed apical constriction [145]. Apical constriction functions to guide tissues into many shapes such as folds, pits, and tubes [146,147]. In addition, several important developmental checkpoints require this process, as illustrated by neural tube closure and mesoderm invagination during gastrulation in *Drosophila* [146,148]. Actomyosin contractility also plays roles in other aspects of embryonic development, such as cell

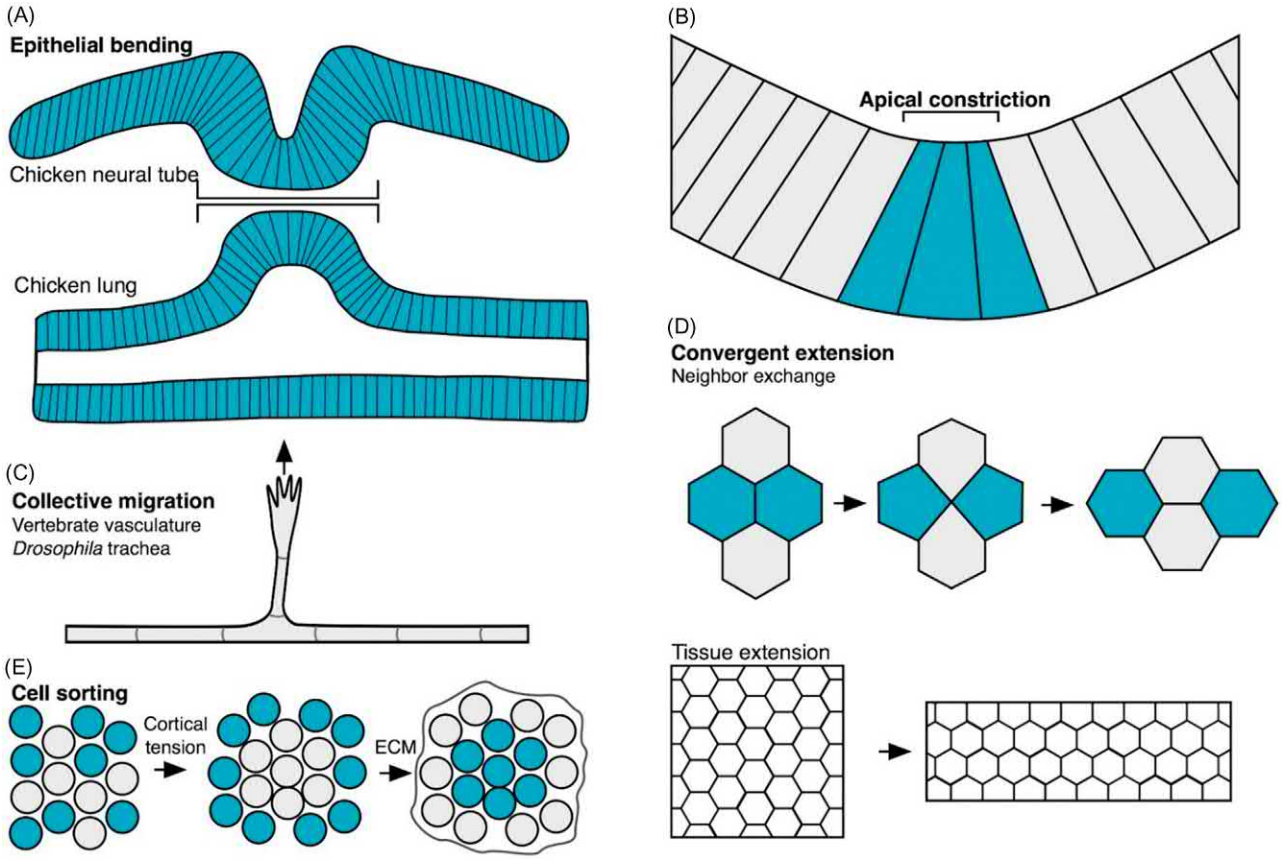

FIGURE 14.8 Mechanical interactions during tissue morphogenesis. Epithelial layers bend (A) during morphogenesis of the neural tube and lung in chicken embryos. This bending, termed apical constriction, can be initiated by actomyosin contraction of the apical epithelial layer of epithelia (B). Cells can also collectively migrate to form lumens in the vertebrate vasculature and *Drosophila* trachea (C). During tissue extension (D), cells rearrange the junctions with their neighbors to generate compaction in one direction and extension in another. Finally, cells can segregate themselves into discrete populations through cortical tension, differential binding among different cell types, and cues from the extracellular matrix (E). *Adapted from Roper K. Integration of cell−cell adhesion and contractile actomyosin activity during morphogenesis. In: Yap AS, editor. Current topics in developmental biology. Amsterdam: Elsevier; 2015.*

intercalation and collective migration, during elongation along the anterior–posterior body axis [144,149].

As organs begin to develop rudimentary functions, they generate macroscale forces that are sensed by cells within their own tissues and those in nearby tissues. These forces help reinforce differentiation and development into specialized organs [144]. One example is fluid shear stress, which modulates vascular remodeling and pruning in the mammalian yolk sac [150]. Fetal breathing–like movements also generate amniotic fluid flow in the developing lungs, which accelerates lung growth and pulmonary cell differentiation [151].

Other mechanical forces include the tension generated by developing muscles, which feeds back to promote muscle differentiation and strengthen cell–cell junctions [60]. Since bone is mechanically coupled to muscles, these forces also promote morphogenesis and strengthening of developing bones [152]. Together, these physical forces act to guide developing tissues toward proper morphogenesis and development.

Cancer metastasis

Cancer morbidity is closely associated with cell migration away from the primary tumor site and establishment of metastases in distal locations termed metastasis. For effective intervention of metastasis, it is important to understand how cancer cells navigate the complex environments. Recent studies have highlighted the importance of mechanical cues, such as matrix stiffness, topology, confinement, shear stress, and mechanical stretching, in metastatic behavior [153].

Tumors are mechanically distinct from normal tissues. They are typically stiffer, due to both an abnormally high concentration and alignment of ECM proteins [154] and the transformation of surrounding fibroblasts, termed cancer-associated fibroblasts, into myofibroblasts [155]. In addition, leaky vasculature from faulty angiogenesis causes an increase in interstitial fluid pressure, which is sensed as tension and shear flow within the tumor [156]. These mechanical cues are known to affect cancer behavior, albeit in a varied manner. Mammary cancer cells metastasize more successfully on stiffer substrates, while other cancer cells appear to lose the ability to respond to variations in stiffness [157,158]. The mechanical stretching induced by fluid pressure causes increased proliferation of some cancer cells through the YAP/TAZ pathway, while other cancer cells lose their responsiveness to shear flow [119]. The varied cancer cell responses to mechanical cues, paralleling their equally varied origins [153], represent one of the major challenges in cancer treatment.

Metastasis initiates with migration of tumor cells away from the primary tumor. The migration is facilitated by the concurrent migration of surrounding cancer-associated fibroblasts, which leave behind trails of highly aligned collagen and fibronectin fibers (Fig. 14.9). The stiffness of these ECM trails may further enhance the migration of tumor cells away from the primary tumor with a high persistence and velocity [159–161]. Distinct mechanical characteristics of tumor cells may facilitate their preferential invasion in certain tissues. For example, migration through the dense bone matrices may be facilitated by a deficiency of nuclear lamin A/C, which manifests as a thinner nuclear lamina, weakened nuclear lamina, and increased deformability of the nucleus to allow cells to squeeze through very small pores that are otherwise impassable to normal cells [162,163].

Cancer cells must navigate through the vasculature and establish themselves at new locations in order to successfully metastasize. Recent studies have suggested that clusters of cancer cells can leave the primary tumor and migrate in a collective fashion to establish metastases [165]. Observed in the circulation, these circulating tumor clusters have been linked to greater numbers of distal metastases and increased morbidity [166]. It is possible that the mechanical strength and integrity of cell–substrate and cell–cell adhesions of cell clusters generate favorable microenvironments to aid the survival of tumor cells [167–169]. Evidence further suggests that these clusters can rearrange themselves into linear trains to pass through thin capillaries and remain within the circulation, which allows them to establish themselves in other areas of the body instead of merely downstream of the circulation [169].

From the initial survival within the primary tumor site to successful metastasis, the behavior of tumor cells appears to be intimately associated with the mechanical cues they experience. Complete understanding of these processes will allow the development of new therapies to stop the uncontrolled spreading of malignant cells, particularly those from highly invasive cancers such as melanoma.

Mechanobiology in tissue engineering

A thorough appreciation of mechanotransduction mechanisms will also facilitate new engineering applications that require seamless integration with biological systems. It is now realized that mechanical incompatibility represents one of the major causes of failure of procedures or devices involving cell interactions. Recent advances in mechanobiology have paved the road leading to novel techniques and therapeutic developments. In this section, representative examples of tools and therapies that are reliant on an in-depth understanding of mechanobiology are highlighted.

Bone-implant design

Artificial implants are used to recapitulate bone and joint function in dentistry and orthopedics. These implants are

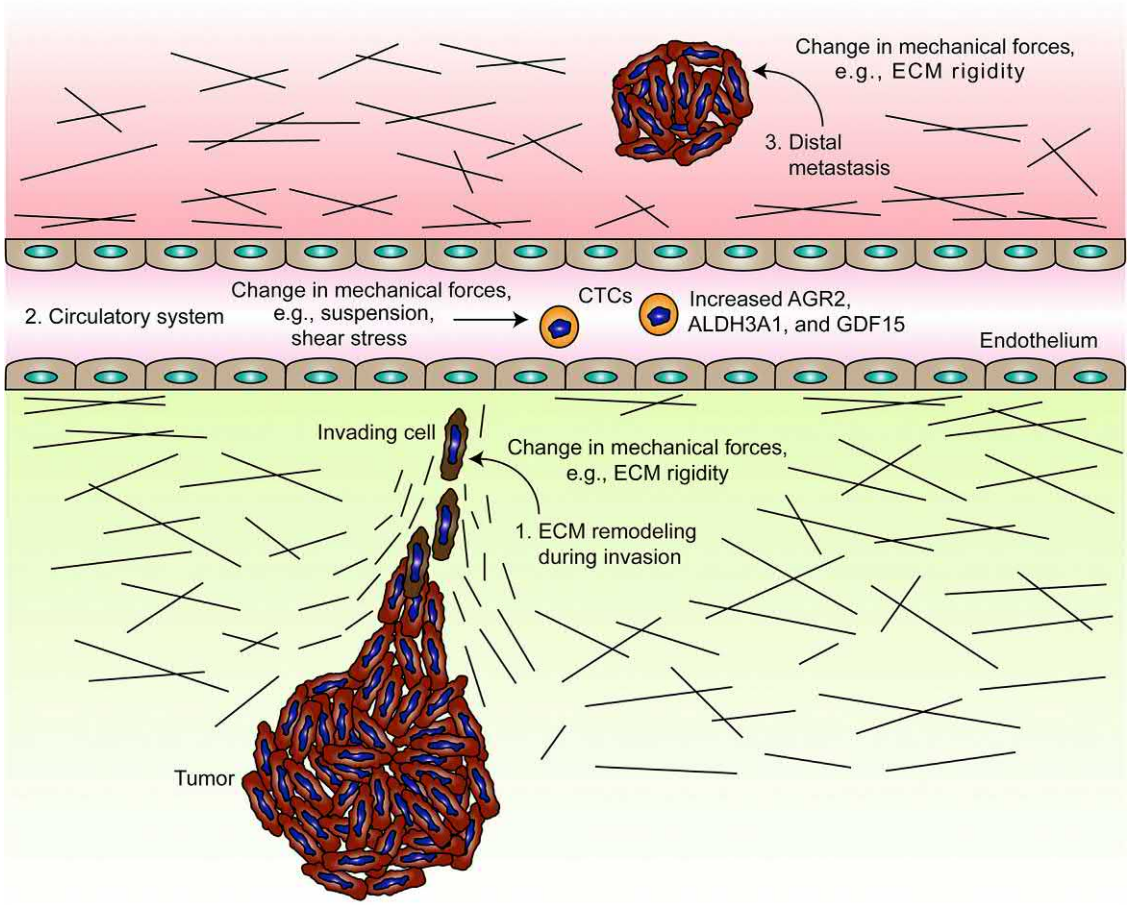

FIGURE 14.9 **Mechanical processes involved in cancer metastasis.** Cells of primary tumor and cancer-associated fibroblasts migrate out and remodel the local extracellular matrix, forming tracks of aligned collagen and fibronectin that promote further migration away from the tumor. After intravasation to enter the blood stream, these circulating tumor cells and clusters may use their actomyosin cytoskeleton and cell–cell adhesions to survive the shear flow and narrow constrictions in the circulatory system. The cancer cells then extravasate and establish secondary metastases, preferentially at sites that have mechanical properties ideal for their proliferation. *Adapted from Aw Yong KM, Sun Y, Merajver SD, Fu J. Mechanotransduction-induced reversible phenotypic switching in prostate cancer cells. Biophys J 2017;112(6):1236–45 [164].*

often made of rigid metals, such as pure titanium or titanium alloys, fastened to native bone [170,171]. After the initial implantation, the stability of the implant is determined by the ability of the body to grow and sustain normal bone structure around the implant in a process called osseointegration [172].

Osseointegration of bone implants requires both bone formation and bone resorption. The same mechanical cues that drive native bone remodeling turn out to also drive the stabilization of artificial bone implants. Native bone is subjected to cycles of compression during movement, which cause osteoblasts and osteoclasts to increase bone formation and decrease bone resorption, respectively [9]. Several techniques have been developed to promote osseointegration, such as coating the surface of the metal with hydroxyapatite, the main chemical component of the bone [173]. Both direct cyclic loading of the implant and loading the entire bone-implant composite have been shown to significantly improve the formation of bone at the implant interface, suggesting that mechanical cues may be used in conjunction with conventional pharmacological treatments to promote osseointegration [170]. These cues could be delivered through a surgical implant, through physical therapy or novel noninvasive therapies, such as whole body vibration [174].

Despite these efforts, the effective lifetime of artificial bone implants remains lower than desirable. Although mechanical loading of the implant promotes bone formation, bone resorption still occurs near the interface [170,175]. Currently, the metals used in these implants are at least an order of magnitude stiffer than the bones they are meant to replace [175,176]. In addition, the implants do not possess the same variations in stiffness that are present in native bone architecture [176]. These differences expose bone structures surrounding the implant to different mechanical conditions from those

seen in native bones, which leads to abnormal patterns of bone formation and resorption and weakening of the bone around the implant (Fig. 14.10) [175,177]. To mitigate these effects, implants are being designed that have comparable architecture and stiffness to native bones [178–180], based on the understanding that decreasing stiffness also tends to increase interfacial stresses between the bone and the implant to cause bone resorption [175]. To promote osseointegration and long-term stabilization of the implant, implants should present the same pattern and magnitude of mechanical signals to the surrounding tissue as the native bone [178–180].

Organs-on-a-chip

Organs-on-a-chip are microfabricated devices designed to recapitulate the function and morphology of an organ. The prevalent experimental models used in biological studies and drug discovery have been 2D culture systems and animal models. Conventional 2D culture systems, such as flat monolayers on tissue culture polystyrene, are inexpensive but fail to capture many of the physiological environmental cues that are important for generating the characteristics of cells in living organs. These spatiotemporal cues include mechanical, chemical, and electrical signals. In contrast, animal models are expensive and often overly complex for the purpose. The results from animal models also fail to apply to human patients in many cases due to cross-species differences. The organ-on-a-chip approach is intended to address the shortcomings of these conventional model systems by recapitulating many of the native environmental characteristics through the placement of cultured cells in microfabricated constructs [183].

A number of such organ-on-a-chip devices have been designed as models for organs, including bone, brain, blood vessels, and lungs [184–187]. In the process of recapitulating the native environment for each organ, several techniques have been developed to reproduce the respective mechanical cues (Table 14.1). For example, the native stiffness in the model organ is recapitulated by adjusting the stiffness of the polymeric materials used in the chip. Notable polymers used in organ-on-a-chip designs are polydimethylsiloxane and polyacrylamide; both have tunable stiffness depending on the composition of monomers and cross-linkers [91,188,189].

To recapitulate organs with cyclic patterns of tension, the device may incorporate cells cultured on flexible membranes undergoing cyclic stretching, which are stretched by applying vacuum to the compartment opposite from the cell [187,190]. A similar approach has been taken to recapitulate the cyclic patterns of compression that the musculoskeletal system are subject to, by using hydrodynamic expansion to exert compression on cell culture chambers [191].

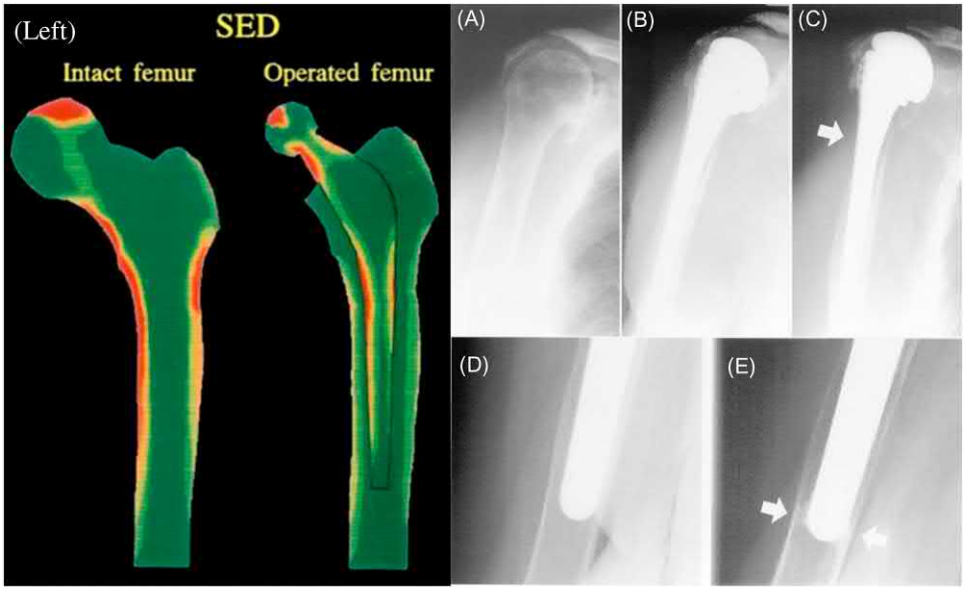

FIGURE 14.10 Stress shielding and abnormal bone remodeling resulting from unmatched mechanics of bone and implant. The strain energy density profile of bone tissue (left) in the proximal femur is drastically altered by the placement of a femoral stem implant, as shown here through finite element modeling of the native proximal femur and the composite after implantation. Differences in bone mechanics are felt by osteoblasts and osteoclasts in the bone tissue as differences in compression and shear flow. This results in abnormal patterns of bone resorption (A–C, *arrow* indicates site of bone resorption) and bone formation (D–E, *arrows* indicate site of bone formation) around the implant that are predicted by the differences in mechanics from finite element modeling. *Adapted from Sumner DR. Long-term implant fixation and stress-shielding in total hip replacement. J Biomech 2015;48(5):797–800; Nagels J, Stokdijk M, Rozing PM. Stress shielding and bone resorption in shoulder arthroplasty. J Shoulder Elbow Surg 2003;12(1):35–9 [181,182].*

TABLE 14.1 Mechanobiological approaches for cell manipulation in organ-on-a-chip devices.

Mechanical cue	Organ culture	Cell type	Environment
Interstitial flow	Vasculature	Primary	3D
Interstitial flow	Brain	Primary	3D
Interstitial flow	Liver	Primary	3D
Substrate stiffness	–	Cell line	2D
Electromechanical	–	Primary	2D
Shear stress	Vasculature	Primary	2D
Shear stress	–	Cell line	2D
Shear stress	Blood–brain barrier	Cell line	2D
Shear stress	Aortic valve	Primary	2D
Shear stress	Blood–brain barrier	Primary	2D/3D
Shear stress	Blood–brain barrier	Cell line	2D/3D
Shear stress	Extravasation	Primary	3D
Shear stress	Vasculature	Primary	3D
Shear stress	Bone	Cell line	2D
Shear stress	Bone	Primary	3D
Shear stress	Vasculature	Primary	2D
Stretching	Lung	Primary and cell line	2D
Stretching	Gut	Primary and cell line	2D
Stretching	Heart	Primary	2D
Stretching	Muscle	Primary and cell line	2D
Stretching	Vasculature	Primary	2D
Stretching	–	Primary	3D
Stretching	Heart	Primary	3D
Stretching	–	Cell line	3D
Stretching	–	–	2D/3D
Stretching	Artery	Primary	2D
Compression	Bone	Primary	2D
Compression	Vasculature	Primary	2D

Dashes (–) indicate devices without a defined target organ, tissue, or cell culture.
Source: Adapted from Ergir E, Bachmann B, Redl H, Forte G, Ertl P. Small force, big impact: next generation organ-on-a-chip systems incorporating biomechanical cues. Front Physiol 2018;9:1417.

When designing microfabricated devices to recapitulate the vasculature or interstitial tissue, it is necessary to recapitulate the fluid flow profiles that cells experience [192]. Model vasculature may be fabricated by seeding cells on the inner wall of a microfluidic channel. For interstitial tissues, cells may be seeded in a 3D scaffold perfused with fluid. Complex flow profiles may be reproduced through model vasculature or interstitial tissues using programmable flow pumps to create steady or pulsatile flows. In addition, laminar or turbulent flow profiles have been created by tuning the microfluidic channel tortuosity [193,194]. Computational fluid dynamics simulations have been used to help predict the exact fluid flow for a given microfluidic design or pump setting to allow rapid design and optimization [195].

Compared to cells without proper mechanical cues, cells cultured on systems incorporating optimal mechanical and chemical features display behaviors more closely

mimicking those in vivo, including proliferation, adhesion, and differentiation [1,183,187,190,191,194]. Much advancements are expected as the field of tissue engineering develops increasingly sophisticated approaches and devices to recapitulate critical features of physiological conditions.

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Part Three

In Vivo Synthesis of Tissues and Organs

Chapter 15

In vivo engineering of organs

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Introduction

Organogenesis is a phase during embryonic development that commences at the end of gastrulation and continues until adulthood and encompasses the process of organ formation from germ layers. Organogenesis requires intricate orchestration of transcription factors and signaling pathways and is under the control of spatiotemporal gradients of trophic factors, chemokines and cytokines. While some organs (e.g., skin, liver, and bone) and tissues (epithelial barriers) are capable of repairing and restoring cellular and physiological homeostasis, most tissues and organs are incapable of repairing following extensive injury as many of the signaling paradigms and regenerative processes that are active during development are not accessible in adulthood. Therefore the key to in vivo engineering of tissues lies in not only identifying cell populations that have pluripotency or capability of restoring the cellular complexity of a tissue (or organ) but signaling molecules that can drive cellular organization and homeostasis. Unfortunately, invoking such processes within the vicinity of diseased or damaged organ poses several challenges, including compromised vascularity, extensive loss of tissue volume, and importantly overcoming degenerative processes (soluble signals, signaling pathways) that are prevalent immediately following insult or disease onset.

Conceptually speaking, tissue engineering can provide a blue-print for organizing cells into functional units. However, the plurality of cells, each with their own unique signaling microenvironment and metabolic demands; and complexity of cellular organization for example in the liver, intestinal crypt, bone can never be faithfully replicated through a conventional tissue engineering paradigm. So, the engineering of tissues with high degree of cellular organization, vascularity, and innervation requires a more radical approach, the one that leverages the innate processes of repair and regulation found within the mammalian body to promote complexity from a well-defined (or a simplified) starting point.

Historical context

One could argue that the ultimate objective of tissue engineering as a discipline is to gain the technical competency to be able to invoke and control biological processes "on demand" and more specifically in a manner so as to drive regeneration and healing. Our own quest in the mid-late 1990s for developing a new paradigm for engineering complex tissues in vivo was motivated in an environment enriched by many successes in the laboratories of pioneers in the Cambridge and Boston area such as Prof. Langer, Prof. Vacanti, Prof. Bell, Prof. Yannas, and Dr. Bruke. For example, Yannas and Burke showed for the first time that a biomaterial with defined physiochemical characteristics could orchestrate the recruitment and organization of cells into a complex structure such as the skin [1–3]. This led to the first skin regeneration product that is still on the market. Incidentally, skin is the largest organ and one could argue that this was the first attempt to de novo engineer a tissue. Langer and co-workers had shown that cardiac myocytes when associated with a polymer scaffold can spontaneously contract and exhibit electrophysiology similar to native cardiac tissue [4]. These efforts solidified the tenets of tissue engineering (also referred to nowadays as regenerative medicine to place it in a broader context) and laid down the well-accepted approach of engineering tissue constructs by marrying concepts from materials science with cell biology, biological process engineering, and clinical-use parameters. During this phase, a huge emphasis was placed on scaffold design [5,6] and developing bioreactors [7] to control cellular environments based on a simplified notion of what a cellular microenvironment should encompass,

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and it resulted in an ad hoc approach of incorporating fluid dynamics, soluble signal (growth factors, proteins) delivery, and biomechanical cues to control cell function. Concurrently, the access to totipotent cells and pluripotent cells [mouse and human embryonic stem cells and mesenchymal stem cells (MSCs)] solidified the narrative that ex vivo engineering of any issue type was within reach [8,9]. But time would tell that this conclusion was rather premature, and alternative approaches to engineering tissue are even more critical today than it was two-and-half decades ago. Nonetheless, in recent years there have been a few notable successes such as joint resurfacing using cartilage engineered from nasal chondrocytes [10]; however, the transplantation of engineered organs such as bladder remains close but elusive [11].

As stem cell populations were being identified, ways to control their phenotype became a prominent scientific endeavor, and the value of traditional 2D tissue culture in this effort was being questioned. Three-dimensional (3D) environments readily accessible with the tools of tissue engineering became therefore highly attractive. Studies showing lineage-specific differentiation of MSCs [8] and trans-differentiation of primary tissue-derived cells [12–14] (of which today there are numerous examples [15–17]) clarified the role for 3D culture in leveraging the benefits ushered in by stem cells. However, lack of access to reproducible and well-defined population of stem cells, and time consuming and costly isolation procedures, difficulties in getting cells to organize into anything with the semblance of tissues were seen as huge limitations. For example, although liver stem cells were identified and isolated [18–21], reconstituting liver-like structures from these cells was met with little success and the best outcomes were still attained by only using freshly isolated hepatocytes.

In the case of bone, a highly vascularized and innervated organ with a complex cellular composition and intricate architecture, bone cells (osteoblasts) alone are not sufficient to achieve this cellular complexity as vascularization and innervation which are central to the formation and structure of bone are not realizable in vitro. In this backdrop, many viewpoints emerged questioning the merits and demerits of synthetic materials as a scaffold versus biological materials such as collagen, the role of ex vivo manipulation and nurturing of tissue constructs using bioreactors versus leveraging innate biologically relevant repair paradigms (and processes), and also how much of engineering was necessary.

Nature's approach to cellular differentiation and organization

During development, the formation of *primitive streak* in the blastula is responsible for the initiation of bilateral symmetry, germ layer formation (i.e., it is in the primitive streak that endodermal and mesodermal progenitors arise) and anterior–posterior symmetry [22] through the out migration and organization of endodermal and mesodermal progenitors eventually resulting in the formation of a second embryonic axis. In fact, during development all mesodermal cells are generated in the primitive streak by epiblasts undergoing epithelial–mesenchymal transition [23,24]. It is well established that the formation of the primitive streak requires well-orchestrated gradients of WNTs, fibroblast growth factors (FGFs) [23,25], bone morphogenetic proteins (BMP 4 and BMP 7), and Nodal a mesoderm inducer from the transforming growth factor (TGF)-beta superfamily of proteins [22]. Likewise, the patterning of the limb buds during limb development commences with the condensation of MSCs into a mesenchyme and the formation of the apical ectodermal ridge, and signaling feedback loops between these two cell populations (epithelial and mesenchymal) leads to patterning and development of limbs [26,27]. So, it is clear that the cellular patterning and organogenesis require precise spatiotemporal control of soluble signals. Current strategies for delivering soluble signals such as FGF-2, vascular endothelial growth factor (VEGF), and BMPs [28], to name a few, depend on either controlled release systems (microspheres, liposomes, and nanospheres) or extracellular matrix–mimicking polymers to electrostatically bind and release morphogens and growth factors, and these have been explored in the induction of new blood vessels (angiogenesis) [29], bone repair, primarily in spinal fusion, and long bone defects where they have received United States FDA approval [30,31] and innervation with varying degrees of success [32,33]. However, signal gradients and pattern in vivo are seldom generated by varying the soluble signals (FGF, BMP, and WNT) but through positive and negative regulators of the soluble signals and their signaling pathway [22,24]. Furthermore, it is now well established that proximodistal and anteroposterior patterning is controlled by an epithelial–mesenchymal feedback loop involving four-dimensional patterning involving positive and negative feedback loops rather than varying morphogen thresholds [34]. Such complex signaling can be realized in part using gradient generating systems [35]; however, implementing such systems in vivo is a tall order. Another aspect of soluble signal delivery is that the responsiveness of cells to soluble signals can vary drastically. For example, while epithelial cells show saturation in WNT pathway signaling with increasing WNT3a concentration, MSCs show no such tendencies [35]. Also, delivery of a single or a cocktail of morphogens can lead to unintended consequences that may be difficult to reverse once initiated. So, it is clear that conventional approaches are less practical and therefore endogenous means of invoking and controlling signal gradients should be the way forward.

It is within this evolving landscape, an idea was conceived as to how one might approach this challenging task of engineering an entire organ in vivo by bypassing many of the steps (cell isolation and expansion, culturing in bioreactors, scaffolds and growth factor delivery) involved in traditional tissue engineering. This concept was called the in vivo bioreactor (IVB).

Conceptual framework of the in vivo bioreactor

Biological systems are inherently noisy [36], and this noise stems from thermal fluctuations that can alter protein structure, receptor−ligand binding rates, diffusion and the fact that activating a kinase can lead multiple proteins being phosphorylated, and they might in turn have more than one binding partner [36,37]. Randomness leads to probabilistic rather than linear outcomes in signaling. For example, if a niche is physically limited, only a few cells can reside in the niche and as a result the outcomes are governed by chance (roll of a dice). So, one way to impose order over chaos is to introduce a strong global cue, something that will synchronize the immediate cellular microenvironment. Hence, in a noisy environment, for a biological information to stand out, it has to be either super specific or overwhelming. That is, knowing which signal is important and how to boost it would be critical to drive the signaling cascade [38].

Therefore the cornerstone of the IVB concept is that biological systems are inherently noisy and hence even a single, but appropriate signal, could completely alter the entire signaling landscape. While, in biology, the signals that are considered relevant are biological in origin (growth factors, proteins, enzymes, RNA, microRNA, virus), the source of signal in the IVB paradigm can be biophysical in nature or can couple to the cellular microenvironment through *mechanosensing* elements to initiate a mechanobiology paradigm and is introduced from the outside into the cellular environment. Such signals (or cues) originate in the physiochemical attribute of intervention and thus ensures that this signal is localized, readily replicated and also finite (in quantity and time).

The challenge is in identifying a set of conditions under which cellular signaling can be controlled in a defined region so as to drive predetermined outcomes. The following four criteria are deemed necessary for the IVB paradigm to be operative and viable (Fig. 15.1):

1. Identifying a location (ectopic or orthotopic) in the body with an inherent source of pluri- or multipotent cells or progenitor cells.
2. Establishing a privileged microenvironment that excludes other cell populations.
3. Presenting a single biophysical or soluble cue that can overcome stochastic biological noise.
4. Defining a volume for the regenerative process.

At the time of the conception of the IVB, it was envisioned that a successful outcome would for the first time present an approach for an autologous solution for organ and tissue transplantation and addressing the core objective of tissue engineering.

Based on the above principles, thus far three distinct cellular organization processes leading to organ and tissue formation have been realized and are discussed here. Furthermore, the opportunity to explore the IVB paradigm in liver regeneration and restoring cellular and signaling homeostasis in brain tissue is presented.

In vivo bone engineering—the bone bioreactor

The first example of the IVB was the bone bioreactor published in 2005 [39]. Engineering bone has always been challenging and much of the effort in the late 1990s and even today has focused on a cell implantation approach, involving isolation of stem cells, preferably marrow-derived stem cells (MSCs), ex vivo expansion, and then associating cells with a scaffold (polymer or ceramic) and implantation of the cell laden scaffold at the site of injury. This approach basically fails to address a key aspect of osteosynthesis, which is vascularization and has to precede bone formation [40−42]. A decade and a half later, there is clear evidence that organotypic vasculature is critical for organ homeostasis [43,44]. So, the IVB concept was as much appealing then as it is now.

Bone formation proceeds during embryonic development by two distinct pathways: (1) the direction ossification by osteoblasts depositing matrix [intramembranous ossification (IMO)] and is the pathway by which flat bones such as the facial bones and skull are formed and (2) the conversion of a provisional cartilage matrix deposited by condensing MSCs by infiltrating bone progenitor cells [endochondral ossification (EO)] and is the pathway by which long bones are formed. Long bones have two membranes: the outer membrane called periosteum that faces the connective tissues and the inner membrane called endosteum, which faces the marrow cavity. The periosteum membrane is composed of an outer fibrous layer, which is highly vascularized, and inner *cambium* layer adhered to the cortical bone surface via Sharpey's fibers and rich in cells—the *periosteal cells*—that have many of characteristics of MSCs [45,46]. Interestingly, periosteum has been shown to play a role in fracture healing and necessary for EO process and is the source of many of the signals necessary for bone repair [45,47,48]. The subperiosteal space essentially meets two of the

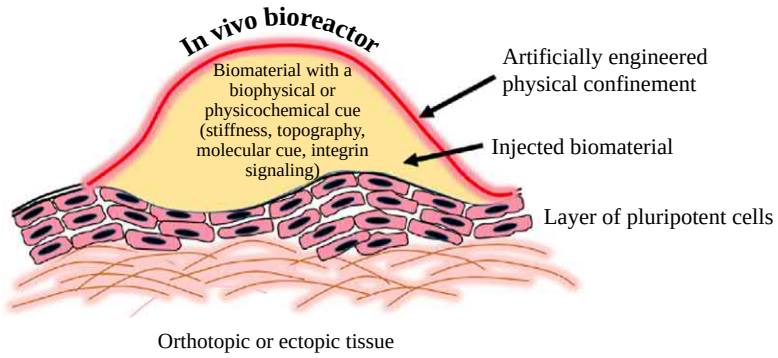

FIGURE 15.1 Schematic showing the various key elements of the IVB paradigm and their crucial roles in ensuring optimal engineering of organ or tissue. *IBV*, In vivo bioreactor.

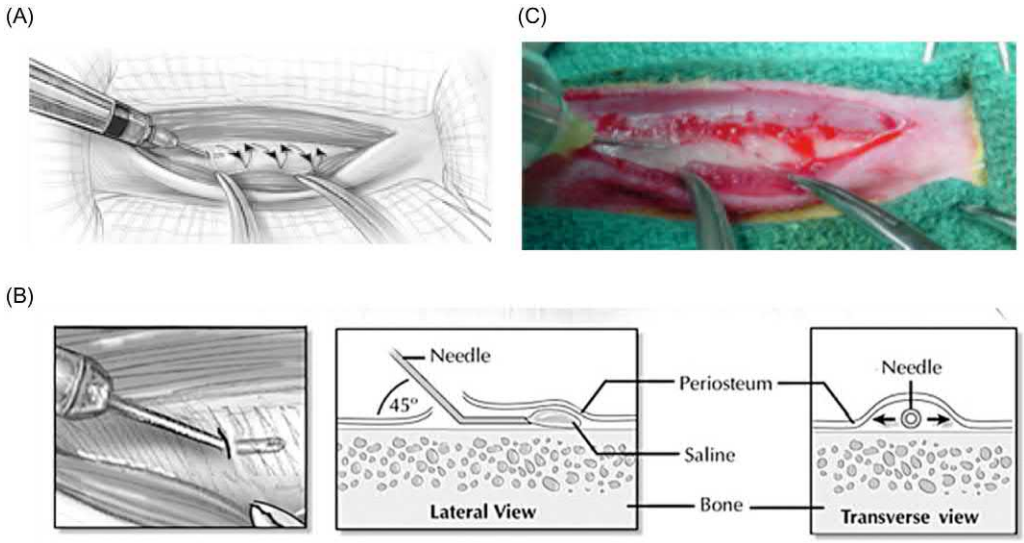

FIGURE 15.2 *Creation of the IVB.* Artist rendition of the periosteal hydraulic elevation procedure (A and B) and photomicrograph showing the needle insertion step in a tibia of a New Zealand white rabbit (C). A 25-ga need bent at an obtuse angle is inserted with the needle axis parallel to the tibial surface and needle opening facing down, thus ensuring that the needle does not pierce through and out of the periosteum. Through fanning motion, a desired breadth of the periosteum can be gently liberated from the underlying cortical bone surface, thus ensuring preservation of the pluripotent cell-rich cambium layer. *IBV*, In vivo bioreactor. *Reproduced with permission from Marini RP, Stevens MM, Langer R, Shastri VP. Hydraulic elevation of the periosteum: a novel technique for periosteal harvest. J Invest Surg 2004;17(4):229–33. Illustration by Fairman.*

criteria for an IVB, namely, it is a source of pluripotent cells and endogenous signaling molecules, it can easily be accessed surgically and can serve as privileged environment if properly manipulated. Surgical manipulation of periosteum is done using periosteal elevators, which are chisels that are used to peal the periosteum back from the cortical bone surface. This procedure, in addition to running the risk of damaging the bone surface and the cambium layer, is also invasive and moreover the area below the peeled back periosteum would be accessible to other infiltrating cells that could impede the healing response. So a new surgical technique called the *hydraulic elevation* was developed [49]. In this novel approach, the subperiosteal space is accessed using a needle inserted at an obtuse angle and Ringer's solution or saline is introduced under hand-pressure using a syringe attached to the needle and the desire area to be lifted is defined by varying the lateral position and the penetration of the needle (Fig. 15.2). This gentle method essentially lifts the periosteum from the cortical bone surface while preserving its structural and cellular integrity [49]. Periosteal explants obtained using this approach were shown to be fully viable in organ cultures ex vivo [50]. This approach ensured that the elevated periosteum represented a confined and privileged space for the introduction of the signal, and the volume for generation of neotissue could be easily defined by injecting a hydrogel.

Although periosteum is rich in growth factor and cytokines, insult to periosteum always results in chondrogenesis. Now the question was what could be an ideal soluble signal to introduce to influence periosteal cell fate? We theorized that, since in EO process, hypertrophy in chondrocytes and matrix calcification precedes bone formation, a calcium (Ca)-rich microenvironment might tip the

balance away from the natural tendency of periosteal cells to undergo chondrogenesis and toward bone formation. Interestingly, calcium can have proliferative effect on cells at certain concentrations [51] and calcium-related signaling events are essential for proliferation and organization of endothelial cells (ECs) [52,53]. Alginate gels that have a long history in cell encapsulation are a natural source of calcium, as calcium is necessary to induce ionic-crosslinking to form the gel. So, in theory, an injection of Ca-alginate should be sufficient to introduce a strong signal—calcium—within the confined subperiosteal space. To our surprise, injection of Ca-alginate within the subperiosteal space in the tibia of skeletally mature rabbits not only induced rapid proliferation of periosteal cells but also induced angiogenesis and exclusively direct bone formation with very-to-little cartilage matrix (Fig. 15.3A) [39]. Essentially, the formation of new bone within the bone IVB had all the hallmarks of IMO and recapitulated a textbook process for osteosynthesis (Fig. 15.3B) and over a 6-week period matured to exhibit microstructure similar to cortical bone (Fig. 15.3C). Importantly, the bone engineered within the IVB could be harvested without damage to the tibial surface and transplanted into a contralateral tibial defect where it underwent remodeling and fusion with the tibia. In validation of the IVB concept, a dominant signal can override stochastic biological noise; growth factors that can enhance bone formation were introduced but had no effect on the quality or quantity of new bone formed [39]. *To date, the in vivo bone bioreactor remains the only example of autologous bone engineering purely through the judicious choice and placement of a biomaterial without any cell transplantation or exogenous supplementation of growth factors.*

The formation of neobone within the IVB is counter intuitive for the following reasons. One, it does not obey Wolff's law which is, bone forms in response to load along lines of mechanical stress [54], and the bone formation in the IVB occurs from the periosteum that is not physically connected to the tibia and hence does not perceive any load from the tibial axis; second, as stated earlier, repair of long bones involving periosteum occurs through EO pathway, yet here in the bone IVB paradigm direct deposition of bone from periosteal cells that had undergone differentiation to osteoblasts was observed, that is, essentially an IMO pathway of bone formation. This confounding observation can be rationalized on the basis of a recent study by Sarem et al., where it was shown that high concentration of calcium results in hyperstimulation of extracellular calcium sensing receptor (CaSR) in MSCs, and this in turns leads to a massive downregulation in the expression of parathyroid hormone

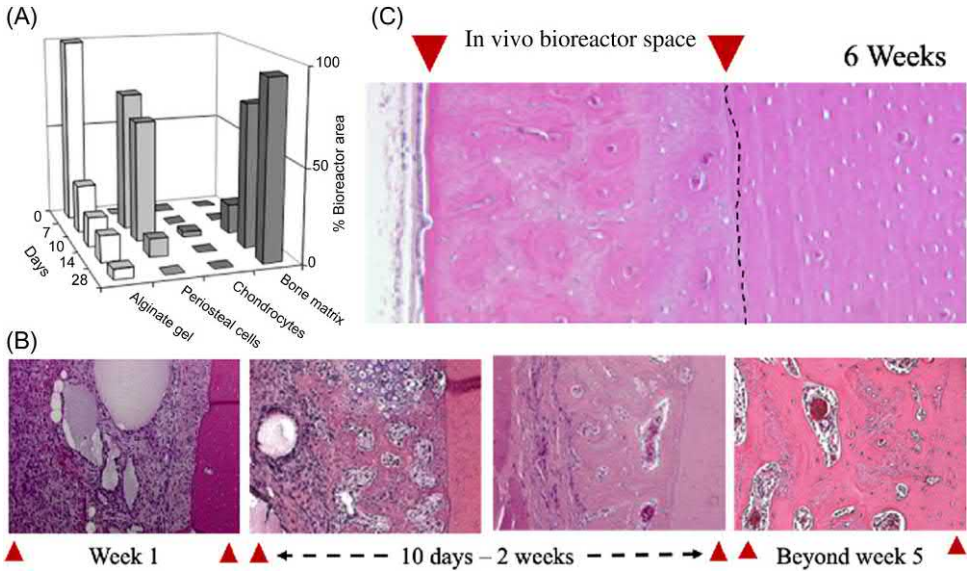

FIGURE 15.3 *Progression of osteosynthesis in bone IVB.* Graphical representation of the changes in cellular composition and resorption of alginate biomaterial over a 4-week period (A). The first 2 weeks are dominated by the proliferation of periosteal derived stem cells, followed laying down of bone matrix by these as they undergo osteogenic differentiation. This process shows clear inverse correlation with degradation of alginate. The absence of any significant population of chondrocytes confirms bone formation through the intramembranous ossification pathway. Hematoxylin–eosin stained sections of the IVB over time showing the cellular events and tissue remodeling occurring within the IVB over a 4-week period (B). Hematoxylin–eosin stained cross section of the IVB and the adjacent native tibia showing the maturation of bone and formation of cement lines and bone lacunae (C). The dashed line demarcates the native bone surface from the IVN environment. Note the absence of any fusion between the bone in the IVB and the tibial cortical bone. *IBV*, In vivo bioreactor. *Reproduced with permission from Stevens MM, Marini RP, Schaefer D, Aronson J, Langer R, Shastri VP. In vivo engineering of organs: the bone bioreactor. Proc Natl Acad Sci USA 2005;102(32):11450–5.*

(PTH) 1 receptor (PTH1R), which is critical for MSC-chondrogenesis [55]. Furthermore, it was shown that the exposure of MSCs to synthetic PTH could reverse the effects of the CaSR−PTH1R differentiation axis in MSCs and partially rescue MSCs from entering osteogenic differentiation [55]. This finding could provide an additional strategy to control periosteal cells fate choices within the IVB environment through administration of agents that modulate PTHIR signaling.

In vivo cartilage engineering

Since periosteal cells can give rise to both osteoblasts and chondrocytes, we inquired if overwhelming the IVB environment with a molecule that can interfere with angiogenesis can alter the fate of periosteal cells. Suramin is a small molecule inhibitor of the TGF-beta superfamily of proteins [56] with a known role in interfering with blood vessel formation [57,58]. To remove calcium as a signal in the IVB, the alginate gel was replaced with a hyaluronic acid (HA)-based gel and then supplemented with Suramin and TGF-beta1. Within 10 days the IVB space was reconstituted with cartilage as opposed to bone further validating the basic tenets of the IVB paradigm (Fig. 15.4). Interestingly, TGF-beta1 administration alone and the HA gel by itself did not promote cartilage formation, suggesting that inhibiting angiogenesis was the key. However, Suramin as a signal to alter periosteal cell fate would never be clinically viable due to serious toxicity issues. We therefore explored if the inherent physicochemical properties of a biomaterial could be used to trigger chondrogenesis within the IVB. We hypothesized that a biomaterial that could restrict, inhibit, or interfere with vascular ingrowth could also yield similar outcomes as Suramin, by promoting hypoxia. Nonetheless, whether hypoxia would alter periosteal cell fate choice remained to be assessed.

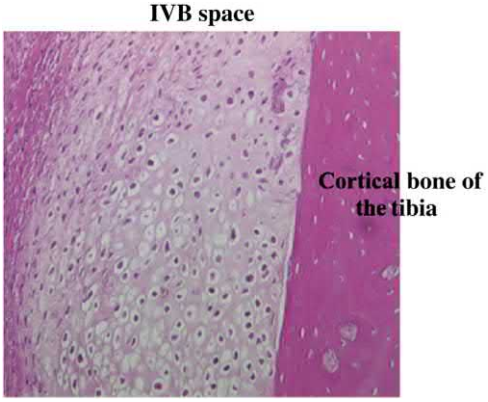

FIGURE 15.4 *The formation of cartilage in the IVB through inhibition of angiogenesis using Suramin.* Hematoxylin−eosin stained cross section of the IVB showing the presence of chondrocytes in the IVB space and the active proliferation of the cells from cambium. *IBV*, In vivo bioreactor. *Reproduced with permission from Stevens MM, Marini RP, Schaefer D, Aronson J, Langer R, Shastri VP. In vivo engineering of organs: the bone bioreactor. Proc Natl Acad Sci USA 2005;102(32):11450−5.*

Agarose, a polysaccharide derived from red sea algae [59], forms physically crosslinked hydrogels at physiological temperature and in vivo. More importantly, studies had shown that agarose is very well tolerated in vivo, and in fact is an inert material with no inherent biological activity, and can undergo degradation [60]. Furthermore, agarose appeared to have no propensity to induce angiogenesis at least in the short term [61]. Based on these characteristics, agarose was identified as the material of choice. Injection of agarose (2 w/v%) in the subperiosteal IVB in the tibia of skeletally matured rabbits resulted in a massive cellular response and the formation of a callus (Fig. 15.5A and B) which by day 21 was made up of hypercellular cartilage (Fig. 15.5C and D) [62]. Not surprisingly, HA gels supplemented with liposomes containing Suramin and TGF-beta 1 also yielded cartilage but offered no superior outcomes in terms of quality or quantity of the engineered cartilage. *The IVB approach remains a singular example to date of in vivo cartilage tissue engineering without cell implantation or growth factor supplementation and is currently being readied for first in human studies.*

In order to ascertain the role of hypoxia in chondrogenesis in the IVB, gene expression levels of hypoxia related factors: hypoxia inducible factor-1alpha and VEGF were quantified using quantitative real-time PCR. A statistically higher mRNA expression was found in tissues from IVB-injected with agarose, thus validating the working hypothesis and rationale for choosing agarose (Fig. 15.5E). A role for hypoxia in the induction of a chondrogenic lineage in periosteal cells was also confirmed in in vitro studies, where a 5−10-fold increase in chondrogenesis associated genes (collagen 2, Sox9, and aggrecan) was observed in cells cultured under hypoxia (0.2% O_2) versus cells cultured under normoxia (21% O_2).

While the engineered cartilage bore no resemblance to the organization found in adult articular cartilage, the hypercellularity of the engineered cartilage turned out to be important. When transplanted into full-thickness osteochondral defects in the tibia of skeletally mature rabbits the IVB generated cartilage was well integrated laterally and with the subchondral bone, and more importantly did not undergo any calcification even after 9 months. This may be attributed to the active remodeling of the hypercellular cartilage matrix as it strives to achieve cellular homeostasis. Such a process would involve apoptosis of cells and this would release not only signaling molecules, but enzymes and proteases that can actively remodel the extracellular matrix providing new space for deposition of

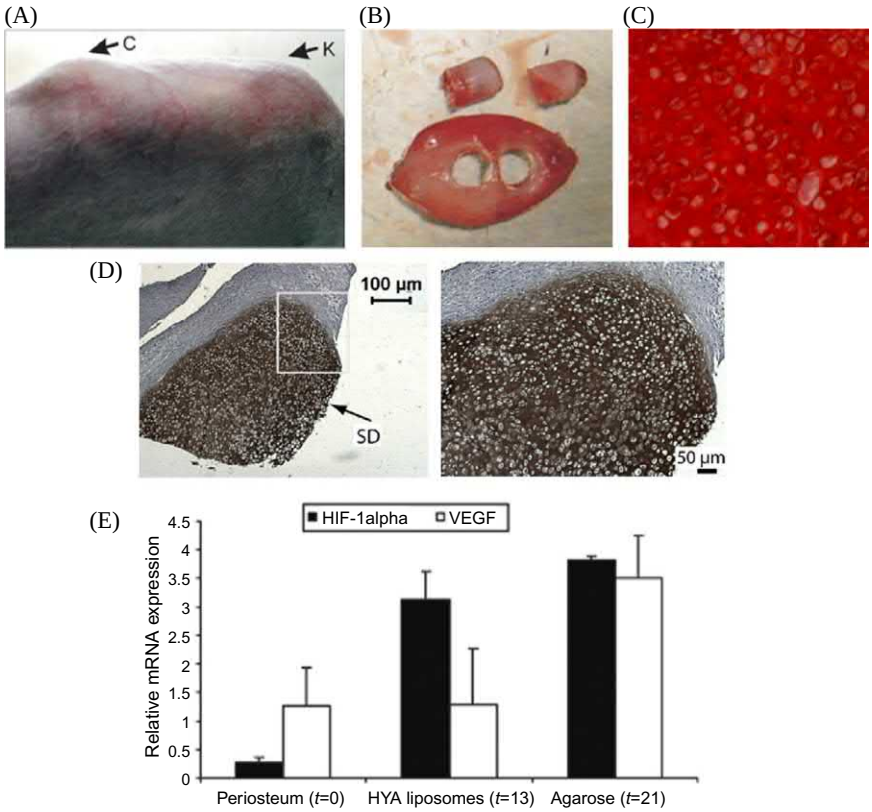

FIGURE 15.5 *Cartilage engineering in the IVB.* Injection of agarose in the IVB in the tibia of a skeletally mature New Zealand white rabbit leads to a string cellular response resulting a callus as early as 7 days which is mature by day 14 (A). "C" Callus, "K" knee joint. The callus is sufficiently large in size and volume to enable coring out of tissue for transplantation is full thickness osteochondral defects (B). Safranin-O stained cryosection of the tissue regenerated within the IVB clearly showing the presence of a glycosaminoglycan rich matrix, a hypercellularity (C). Immunohistochemistry for type 2 collagen, verifying the chondrogenic phenotype of the engineered tissue (D). SD indicates the site of dissection of the cartilage from the IVB. Expression levels of hypoxia related genes HIF-1alpha and VEGF in tissue generated in IVB in the absence of any hydrogel, and when injected with hyaluronic acid supplemented with liposomes containing Suramin and TGF-beta 1 and agarose, showing the marked upregulating of these genes in agarose condition (E). *HIF-1alpha*, Hypoxia inducible factor-1alpha; *IBV*, in vivo bioreactor. *Reproduced with permission from Emans PJ, van Rhijn LW, Welting TJ, Cremers A, Wijnands N, Spaapen F, et al. Autologous engineering of cartilage. Proc Natl Acad Sci USA 2010;107 (8):3418–23.*

new matrix by the chondrocytes. The ability to engineer large volumes of hypercellular cartilage "on demand" can also impact treatment of bone trauma. Ossification is the natural progression of cartilage once it undergoes hypertrophy. Martin and co-workers have exploited this biological process in a bone engineering approach; they have termed *developmental engineering* [63,64]. They have shown that cartilage engineered from marrow-derived MSCs and driven towards hypertrophy can undergo remodeling in vivo into bone and marrow components upon implantation in an ectopic site and using this approach they have also engineered an ectopic marrow with a reconstitute immune system [63–66]. The IVB paradigm therefore, could have far-reaching impact in treating skeletal trauma.

The ability to engineer in vivo, two fundamentally different tissues: *Bone*—which is an organ that is highly vascularized and innervated, and *Cartilage*—which is an avascular tissue; from the same pluripotent stem cell source through judicious choice of an injectable hydrogel was not only a first in exploiting biophysical and physicochemical attributes of biomaterial to influence stem cell fate but was also overwhelming proof that the basic principles of the IVB paradigm were well founded.

Induction of angiogenesis using biophysical cues—*organotypic vasculature engineering*

Angiogenesis is the key to ensuring that any engineered tissue integrates and remodels and achieves cellular homeostasis. Vasculature is the conduit for recruitment of endogenous cell populations [progenitor cells and immune cells (circulating monocytes, B-cells, T-cells, differentiated macrophages M1, M2) [67]]. Equally important is

the emerging evidence that paracrine signaling between ECs and organ forming cells may be the key to morphogenesis, and that vasculature is tailored to a specific organ—*organotypic vasculature*. As discussed earlier, it is now well established that vasculature precedes osteosynthesis and in this regard Adams and co-workers have shown that the type of vasculature is very critical for osteosynthesis [43]. They discovered a new capillary subtype in murine skeletal system that has distinct morphological, molecular, and functional properties, and these capillaries could be differentiated form normal capillaries by the expression of Endomucin (Emcn) as high Emcn expressing vessels and low Emcn expressing vessels, which they have termed H- and L-type vessels, respectively. They also observed an association between H-type vessels and osteoprogenitors cells and osteogenic processes and in aging mice, loss in osteoprogenitor cell population correlated with the loss in H-type vessels and increase in L-type vessels. Furthermore, they have shown that angiocrine signaling, osteosynthesis, and angiogenesis are coupled through EC-specific Notch signaling [44]. While these findings need to be validated in nonhuman primates and in humans, the evidence for a role of vasculature in organ formation is definitely strengthening. In addition, studies from Mooney's group have shown that angiogenesis can also have a positive effect on muscle innervation, formation of neuromuscular junctions, and muscle regeneration [68,69].

Based on this emerging body of evidence, a strong argument can be made that capillary networks are in a sense an organ, perhaps an organoid, as it has cellular and structural complexity, morphology, distinct physiological attributes, which has a role in cellular homeostasis and repair of an organ or tissue, and this goes beyond the conventional notion of a transport function. Engineering of *organotypic vasculature* (or tissue-specific form of angiogenesis) will therefore be the key to successful engraftment of cells and tissues and constitutes a huge unmet need in tissue engineering. Furthermore, transplantation of engineered microvasculature could be one way to induce regenerative mechanisms in vivo.

Angiogenesis, the formation of new blood vessels, is a complex process involving orchestrated released of trophic factors, and paracrine signaling between ECs and perivascular support cells (pericytes). There are several pathologies that are associated with loss of vascularity and vascular function and they include cardiac ischemia, hind limb ischemia, avascular necrosis, and diabetic ulcers. Pioneering efforts by Isner and Asahara in the mid-late 1990s led to the development of a clinical strategy to induce new blood vessels (collateral vessels) in ischemic tissues from existing vasculature using external cues, an approach they called *therapeutic angiogenesis* [70,71]. The idea was to inject a bolus of angiotropic factors such as VEGF or FGF to induce sprouting of existing vasculature and recruitment of resident endothelial progenitor cells [72]. This approach was later expanded to include direct myocardial (tissue) gene transfer using plasmid encoding for VEGF [73,74]. One of the challenges with local delivery of proangiogenic signals is that the sprouting and maturation of new vessels into normal or aberrant (angiomas) depends on local growth factor concentration [75] which is very difficult to modulate and control. ECs also lose their quiescence following injury and get primed toward external proangiogenic cues. However, maintenance (stabilization) of neovasculature is necessary for restoring vascular homeostasis, and in this regard, perivascular cells, that is, pericytes and vascular smooth muscle cells play a critical role in vivo in not only providing scaffolding but also paracrine signaling necessary for blood vessel sprouting and maturation [76]. In therapeutic angiogenesis however, vessels often fail to mature and they regress over time [77] due to inadequate recruitment of appropriate support cells. Therefore, control over cell fate, phenotype and recruitment is key for a successful pro-angiogenic therapy.

Numerous studies have demonstrated that in addition to soluble signals substrate or extracellular matrix stiffness [78], stochastic roughness (nanotopography) [79–81] can dictate cell shape, fate, and function. This paradigm termed—*mechanobiology*—involves invoking and controlling biological processes using mechanical and biophysical cues and represents the next frontier in control of cell function via physicochemical properties of biomaterial. Hydrogels are highly suitable for implementing mechanobiology paradigms as their mechanical properties can be tuned on demand by altering the characteristics of the crosslinks. Carboxylated agarose (CA) represents a new family of polysaccharides that yields physically crosslinked, injectable hydrogels whose modulus can be tuned from 10s of Pa to 10^5 Pa independently of concentration [82,83]. Using CA hydrogels of varying stiffness modified with the integrin binding peptide sequence arginine–glycine–aspartic acid (RGD) and supplemented with angiocrine factors as a screening platform, we discovered that apical–basal polarization in ECs could be induced within soft hydrogels with a modulus around 20–50 Pa [82]. This hydrogel stiffness incidentally corresponds to the mechanical properties of embryonic tissue [84,85]. Under these conditions, ECs migrated and organized themselves into free standing lumens over 100 μm in height comprising 3–5 ECs per lumen cross section. This organization, which is reminiscent of vasculogenesis, that is, the formation of blood vessels from endothelial progenitor cells (which is different from sprouting angiogenesis that is branching from existing vasculature), more importantly occurred in this gel environment in the absence of any support cells. Since CA gels without RGD did not promote polarization of ECs it was deemed that

signaling via integrins and mechanical coupling of ECs to the gel were critical. This demonstrated that apical−basal polarization and organization of ECs into three dimensional structures can be induced purely by matrix mechanics, that is, even in absence of mural support cells.

Encouraged by this observation we inquired if the mere introduction of a biomaterial with defined mechanical properties would be sufficient in promoting maturation and stabilization of neovasculature. Gels of two different stiffness, one with a shear modulus of 0.5 kPa (500 Pa) which is similar to the fibrin network in a blood clot (0.06−0.6 kPa [86]) and the other with a stiffness of 5 kPa which is similar to the gastrocnemius lateralis muscle (E of 11 kPa which corresponds to a shear modulus of around 3−4 kPa [87]), were injected in the mouse gastrocnemius muscle, and the neovasculature formed was characterized for morphological traits [diameter, vessel length density (VLD), and segment length], patency using lectin perfusion index and stability [88]. Both soft and stiff gels were capable of inducing new micro-vessels by 2 weeks, which were physiologically associated with appropriate mural cells, that is, pericytes [positive for nerve/glial antigen 2 (NG2) and negative for α-smooth muscle actin (SMA), and smooth muscle cells (positive for α-SMA)]. However, beyond 7 weeks when vessels become independent of angiocrine factors [75], only the gel environment with mechanically properties similar to fibrin clot was able to promote appropriate branching of the vessels as characterized by short segment lengths and higher VLD (Fig. 15.6A and B) [88]. Furthermore, soft gel environments also promoted long-term stabilization and patency of the newly formed vasculature as seen by the robust association of pericytes with the ECs (Fig. 15.6C). Quantification of pericyte coverage, which was done by computing the ratio of vessel length associated with NG2 + pericytes/total vessel length, revealed that vascular networks within the soft gels had a fivefold greater pericyte coverage compared to those within the stiff gels (Fig. 15.6E). As in the case with bone and cartilage engineered within the IVB, supplementation with either angiocrine factors or basement membrane components bestowed no additional benefits over the gel by itself. The fact that an avascular environment devoid of any inherent angiogenic properties could induce and stabilize new vessels once again confirms that biophysical attributes of a biomaterial alone are sufficient to invoke and control biological processes. The big question now is, how does the gel's stiffness influence vessel stability?

Since proliferation of ECs within the two gels could account for the observed outcomes, explants after 7 weeks were cryosectioned and immunostained for Ki67, a proliferation marker, which excludes quiescent cells [89] and it was found that vascular networks in both soft and stiff gels were essentially quiescent with at least 98% of ECs in the G0 phase (Fig. 15.6D). Having excluded proliferation as a contributing factor myeloid cells and specifically pro-maturative CD11b + monocytes within each gel environment were quantified. We found that the softer fibrin clot like gel recruited 40% higher CD45 + myeloid cells over stiffer gel environment, although the enrichment of CD11b + cells were similar in both gel environments. Further analysis revealed the presence, for the first time, of a hitherto unknown population of CD11b + cells that express the mechanosensing receptor Piezo-1, and more importantly this mechanosensing subpopulation of CD11b + was differentially recruited in the two gel environments, and furthermore, was detected at a higher frequency in the softer than in the stiffer hydrogels, representing 93.1% ± 1.4% of the total CD11b + cells in the soft gels versus 71.8% ± 2.4% in the gels with muscle-like stiffness (Fig. 15.6F) [88]. These findings demonstrated that the biophysical attributes of a hydrogel can also influence the recruitment of immune cells and more importantly provide a bias toward cell populations that are inherently sensitive to mechanical cues. Interestingly, Piezo-1-expressing CD11b + cells is found in the circulation of both mouse and healthy human donors, with surprisingly similar frequency, accounting for 35.0% ± 2.2% and 35.1% ± 9.1% of total CD11b + cells, respectively, and this bodes well for recapitulating the same outcomes in humans. Therefore, in addition to signaling via integrins [90],stretch-activated ion channel Piezo-1, which is also an integrin activating transmembrane protein [91], has a critical role in angiogenesis [92]. This observation when taken in context with the finding that gel stiffness modulates recruitment of CD11b + /Piezo-1 + monocytes provides a strong molecular biology link between the mechanical properties of the hydrogel and blood vessel stabilization and offers a novel direction for future efforts in developing transplantable vascular networks for organ engineering.

De novo liver engineering

In spite of the fact that liver is an organ with the most regenerative capacity, engineered livers have yet to materialize. Although significant progress has been made in producing liver-like organoids for drug screening [93], and there is also evidence that these organoids can expand in volume in an orthotopic site [94], engineering healthy liver tissue on demand remains an unmet need and enormous challenge. One could envision that the principles of IVB could also be leveraged to engineer sizeable amounts of healthy liver tissue at an ectopic site within close proximity to the liver. Anatomically speaking, liver has evolved to compactly fit in the abdominal cavity in close proximity to venous return of the blood to the heart. Therefore creating new volume in this tight cavity is

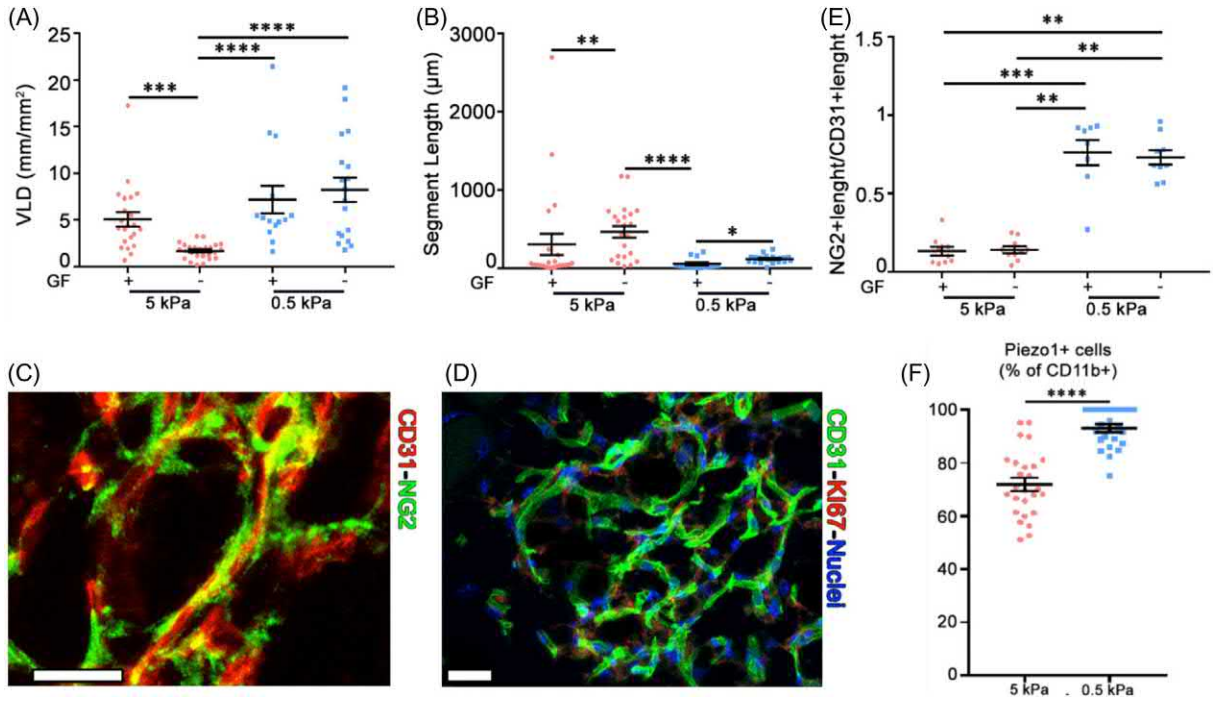

FIGURE 15.6 Morphological characterization of new capillaries formed within soft and stiff RGD-modified carboxylated hydrogels 7 weeks after injection in the gastrocnemius muscles of SCID mice: vessel length density (A), segment length (B), immunofluorescence staining of endothelium in the soft gel (0.5 kPa) (CD31, in red), pericytes (NG2, in green), smooth muscle cell (α-SMA, in cyan) showing intimate tight association of pericytes with ECs (C), immunofluorescent images of soft gels showing the presence appropriately branched and quiescent vasculature (D), pericyte coverage in vasculature within the gels, note the remarkably higher coverage of pericytes in the softer gel environment (E), quantification of recruitment of CD11b + /Piezo-1 + circulating monocytes within the soft and stiff gels (F). Scale bars = 20 μm. $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$ by Kruskal–Wallis test; $n = 4$ independent muscles per each group. *ECs*, Endothelial cells; *SMA*, smooth muscle actin. *Reproduced with permission from Forget A, Gianni-Barrera R, Uccelli A, Sarem M, Kohler E, Fogli B, et al. Mechanically defined microenvironment promotes stabilization of microvasculature, which correlates with the enrichment of a novel Piezo-1(+) population of circulating CD11b(+)/CD115(+) monocytes. Adv Mater 2019;31(21):e1808050.*

extremely challenging. Although like long bones the lobes of the liver are also ensheathed in a capsule of connective tissue called the Glisson capsule this membrane however is too thin (around 20 μm) to be manipulated surgically without causing extensive damage to the liver tissue [95]. Therefore an alternative would be the peritoneal membrane, which covers the liver on the diaphragmatic side and one could envision creating a privileged cavity, fully vascularized, using amniotic membrane (or amnion) as an artificially defined physical barrier—*the in vivo liver bioreactor*. Amnion is appealing as it is easily sourced from discarded placenta and so free of ethical issues, can be processed into a sterile product, has an extensive history of successful use in ophthalmology in corneal reconstruction [96,97] and also appears to be an immune-privileged tissue [98]. One could combine the implantation of organoids with an artificial amnion sac vascularized on the peritoneum as potential means of engineering healthy liver tissues. A key advantage of this approach over, for instance, implanting organoids directly into a liver lobe as explored by Bhatia and co-workers, would be that the evolution of the tissue will be free from the pathology inflicting the recipient. In the cornea, amnion has been shown to promote re-epithelialization as it functions as a basement membrane [97]. Since hepatocytes are epithelial cells one could envision that liver organoids could adhere and proliferate within such environment leading to organogenesis under more physiological conditions. Also, it has been shown recently that epithelial cells from the amnion possess many of the characteristics of hepatocytes, including secretion of albumin [99]. Furthermore, cells from the amnion are capable of differentiating into cells of the three germ layers and using freshly isolated amnion could prove advantageous to exploit this attribute [100]. Volumizing this artificially created amniotic sac (the bioreactor) with a hydrogel that can support rapid vascularization to meet the high metabolic needs of hepatocytes will be the key. Toward this objective the CA hydrogels offer couple of clear advantages, namely, they can be injected as a liquid and set in vivo, thus allowing precise manipulation of the artificial IVB site, and their ability to promote rapid ingrowth of blood vessels from exiting vasculature (sprouting angiogenesis) without the need for any proangiogenic soluble factors.

Repairing brain tissue through controlled induction of reactive astrocytes

Brain tissue is very challenging to repair and restore as it lacks an endogenous repair mechanism. Astrocytes, which constitute more than 30% of all the cells in the brain, are key players in maintaining brain function. Recent studies have shown that astrocytes play a prominent role in formation, function, maintenance, and elimination of synapses [101]. After brain injury and in brain pathology, astrocytes get activated and these reactive astrocytes (RAs) alter cellular and signaling homeostasis. Local delivery of molecules in theory can be used to alter cellular homeostasis in the brain. For example, it has been shown in vitro that treatment of primary murine midbrain cells with FGF and platelet-derived growth factor has a significant positive effect on the survival and maturation of dopaminergic neurons [102]. However, delivery of growth factors to the brain is challenging due to diffusion constraints [103], and therefore using resident cells as sources of soluble signals is more practical. Since RAs can either be neuroprotective or harmful, an alternative strategy is to leverage RAs at various stages of reactivity. Currently, researchers are actively researching how to isolate astrocytes at various reactive states, and if the reactive states of astrocytes can be used as a therapeutic tool in the treatment of CNS diseases and injury [104]. Isolating and manipulating astrocytes ex vivo is very tedious and costly, and standardizing such procedures for clinical use is even more challenging if not totally impractical. In this regard, understanding how to invoke different RA states in vivo and developing minimally invasive strategies to do so could provide a new avenue to repair and restore brain tissue and likely more acceptable clinically.

Blumenthal et al. have shown that astrocytes provide physical cues to neurons, and this is critical for proper neuron function [81]. Furthermore, this interaction between astrocytes and neurons was shown to involve Piezo-1. Loss of Piezo-1 activity in both neurons and astrocytes was found to be detrimental to their function. More interestingly, this study showed that biophysical cues in the form of stochastic nanoroughness has a prominent role in controlling astrocytes phenotype and this provides the basis for an intriguing idea, wherein carefully placed micro-implants of defined topography and/or stiffness can be used to invoke different types of RAs, thus driving local repair processes in the brain. Observations from our ongoing studies in controlling glial scarring round brain implants suggest that such an approach is very much feasible.

Conclusions and outlook

Organ engineering is coming of age as advances in cell and molecular biology, genetic manipulation (CRISPR/Cas9), and materials science coalesce. In this regard, the IVB paradigm presents new opportunities to engineer autologous tissue for transplantation. The advantages of bone and cartilage engineering using the IVB are that quality of the tissue can be assessed noninvasively using MRI before transplantation. In the case of cartilage, the ability to generate a large volume in the neighborhood of $5-10\,cm^3$ (extrapolation based on volumes obtained in rabbits) per injection provides an opportunity to carryout entire articular resurfacing. Furthermore, as discussed earlier, such hypercellular cartilage may also be useful in in vivo engineering of bone through activation of the EO program. Nonetheless, one of the challenges in translating the IVB into the clinic has been the resistance of clinicians to explore unconventional strategies for bone and cartilage repair. Our experience has been that the clinical mindset prevents implementation of solutions that involve two steps (or two procedures), namely, one to create the IVB and the second to harvest the tissue for transplantation, even though the first procedure can be minimally invasively carried out on an out-patient basis. It is anticipated that the first-in-human trials of the cartilage IVB will help in overcoming this perceived downside.

The ability to engineer vasculature on demand will be highly valuable in transplantation of islet cells and also engineering of secondary organ such as a "pancreas on a kidney" or "pancreas in a muscle." The kidney capsule, which is an immune privileged site, meets many of the requirements for an IVB. There has been significant progress to date in transplanting islet cells under the kidney capsule [105]. Kumagai et al. have shown the prevascularized islets can be engineered under the kidney capsule using autologous cells and that such prevascularized islet kidneys are much more efficient than nonvascularized islets at reversing hyperglycemia in swine [106,107]. One can envisage the engineering of a pancreas-specific vasculature and introducing islet cells associated with this vasculature under the kidney as a secondary source for insulin in individuals with type-1 diabetes. Finally, one can also envisage highly engineered implants that incorporate 3D printing with soluble signal gradient generators, for in vivo programming and harvesting of organ-specific cells derived from autologous (patient-derived) induced pluripotent stem cells, essentially turning the human body into a *cell on demand* factory. Such systems could be subdermally implanted for easy access to reprogrammed cells and removal of the implant.

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Part Four

Biomaterials in tissue engineering

Chapter 16

Cell interactions with polymers

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Scaffolds composed of synthetic and natural polymers have been essential components of tissue engineering since its inception [1]. Synthetic polymers are currently used in a wide range of biomedical applications, including applications in which the polymer remains in intimate contact with cells and tissues for prolonged periods (Table 16.1). Many of these polymer materials have been tested for tissue engineering applications, as well. To select appropriate polymers for tissue engineering, it is helpful to understand the influence of these polymeric materials on viability, growth, and function of attached or adjacent cells. In addition, it is now possible to synthesize polymers that interact in predictable ways with cells and tissues; understanding the nature of interactions between cells and polymers provides the foundation to this approach.

This chapter reviews previous work on the interactions of tissue-derived cells with polymers, particularly the types of synthetic polymers that have been employed as biomaterials. Its focus is on the interactions of cells with polymers that might be used in tissue engineering; therefore, both in vitro and in vivo methods for measuring cell−polymer interactions are described. The interaction of cells in flowing blood, particularly platelets, with synthetic polymer surfaces is also an important aspect of biomaterials design, but is not considered here.

Methods for characterizing cell interactions with polymers

In vitro cell culture methods

Cell interactions with polymers are often studied in vitro, using cell culture techniques. While in vitro studies do not reproduce the wide range of cellular responses observed after implantation of materials, the culture environment provides a level of control and quantification that cannot be obtained in vivo. Cells in culture are generally plated over a polymer surface, and interaction is allowed to proceed for several hours; the extent of cell adhesion and spreading on the surface is then measured. By maintaining the culture for longer periods, perhaps for many days, the influence of the substrate on cell viability, function, and motility can also be determined. Since investigators use different techniques to assess cell interactions with polymers, and the differences between techniques are critically important for interpretation of interactions, some of the most frequently used in vitro methods are reviewed in this section.

To perform any measurement of cell interaction with a polymer substrate, the polymeric material and the cells must come into contact. Preferably, the experimenter should control (or at least understand) the nature of the contact; this is a critical, and often overlooked, aspect of all of these measurements. Some materials are easily fabricated in a format suitable for study; polystyrene (PS) films, for example, are transparent, durable, and strong. Other materials must be coated onto a rigid substrate (such as a glass coverslip) prior to study. But since cell function is sensitive to chemical, morphological, and mechanical properties of the surface, almost every aspect of material preparation can introduce variables that are known to influence cell interactions.

Adhesion and spreading

Most tissue-derived cells require attachment to a solid surface for viability and growth. For this reason the initial events that occur when a cell approaches a surface are of fundamental interest. In tissue engineering, cell adhesion to a surface is critical because adhesion precedes other cell behaviors such as cell spreading, cell migration and, often, differentiated cell function.

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TABLE 16.1 Some of the synthetic polymers that might be useful in tissue engineering, based on past use in biomedical devices.

Polymer	Medical applications
PDMS, silicone elastomers	Breast, penile, and testicular prostheses Catheters Drug delivery devices Heart valves Hydrocephalus shunts Membrane oxygenators
PEU	Artificial hearts and ventricular assist devices Catheters Pacemaker leads
PTFE	Heart valves Vascular grafts Facial prostheses Hydrocephalus shunts Membrane oxygenators Catheters and sutures
PE	Hip prostheses Catheters
PSu	Heart valves Penile prostheses
PET	Vascular grafts Surgical grafts and sutures
pMMA	Fracture fixation Intraocular lenses Dentures
pHEMA	Contact lenses Catheters
PAN	Dialysis membranes
Polyamides	Dialysis membranes Sutures
PP	Plasmapheresis membranes Sutures
PVC	Plasmapheresis membranes Blood bags
Poly(ethylene-*co*-vinyl acetate)	Drug delivery devices
PLA, PGA, and PLGA	Drug delivery devices Sutures
PCL	Electrospun materials
PEG	Tissue sealents Surface modification of materials
PS	Tissue culture flasks
PVP	Blood substitutes
Poly(*p*-dioxanone) and poly(omega-pentadecalactone-*co*-*p*-dioxanone) [2]	Sutures

PAN, Polyacrylonitrile; *PCL*, poly(caprolactone); *PDMS*, polydimethylsiloxane; PE, polyethylene; *PEG*, poly(ethylene glygol); *PET*, poly(ethylene terephthalate); *PEU*, polyurethanes; *PGA*, poly(glycolic acid); *pHEMA*, poly(2-hydroxyethylmethacrylate); *PLA*, poly(L-lactic acid); *PLGA*, poly(lactide-*co*-glycolide); *pMMA*, Poly(methyl methacrylate); *PP*, polypropylene; *PS*, polystyrene; *PSu*, Polysulphone; *PTFE*, poly(tetrafluoroethylene); *PVC*, poly(vinyl chloride); *PVP*, poly(vinyl pyrrolidone).

A number of techniques for quantifying the extent and strength of cell adhesion have been developed: a more complete description of these techniques with early references is available (see Chapter 6 of Ref. [3]). In fact, so many different techniques are used that it is usually difficult to compare studies performed by different investigators. This situation is further complicated by the fact that cell adhesion depends on a large number of experimental parameters, many of which are difficult to control. The simplest methods for quantifying the extent of cell adhesion to a surface involve three steps: (1) suspension of cells over a surface, (2) incubation of the sedimented cells in culture medium for some period of time, and (3) detachment of loosely adherent cells under controlled conditions. The extent of cell adhesion, which is a function of the conditions of the experiment, is determined by quantifying either the number of cells that remain associated with the surface (the "adherent" cells) or the number of cells that were extracted with the washes. Radiolabeled or fluorescently labeled cells can be used to permit measurement of the number of attached cells. Alternatively, the number of attached cells can be determined by direct visualization, by measurement of concentration of an intracellular enzyme, or by binding of a dye to an intracellular component such as DNA. In many cases the "adherent" cells are further categorized based on morphologically differences (e.g., extent of spreading, formation of actin filament bundles, and presence of focal contacts). This technique is simple, rapid, and, since it requires simple equipment, common. Unfortunately, the force that is provided to dislodge the nonadherent cells is often not controlled, making it difficult to compare results obtained from different laboratories, even when they are using the same technique.

This disadvantage can be overcome by using a centrifuge or a flowing fluid to provide a controllable and reproducible detachment force. In centrifugal detachment assays, the technique described earlier is modified: after the incubation period, the plate is inverted and subjected to a controlled detachment force by centrifugation. In most flow chambers the fluid is forced between two parallel plates. Prior to applying the flow field, a cell suspension is injected into the chamber, and the cells are permitted to settle onto the surface of interest and adhere. After some period of incubation, flow is initiated between the plates. These chambers can be used to measure the kinetics of cell attachment, detachment, and rolling on surfaces under conditions of flow. Usually, the overall flow rate is adjusted so that the flow is laminar, and the shear stresses at the wall approximate those found in the circulatory system; however, these chambers can be used to characterize cell detachment under a wide range of conditions.

Radial flow detachment chambers have also been used to measure forces of cell detachment. Because of the geometry of the radial flow chamber, where cells are attached uniformly to a circular plate and fluid is circulated from the center to the periphery of the chamber along radial paths, the fluid shear force experienced by the attached cells decreases with radial position from the center to the periphery. Therefore in a single experiment, the influence of a range of forces on cell adhesion can be determined. A spinning disk apparatus can be used in a similar fashion [4], including use with micro- or nanopatterned substrates [5]. Finally, micropipette techniques can be used to measure cell membrane deformability or forces of cell−cell or cell−surface adhesion [6].

There is a growing interest in the interaction of polymer nanoparticles with cells, particularly in the design of nanoparticles that interact with certain populations of cells after introduction into the body. One useful variation in the cell adhesion assay involves flowing polymer nanoparticles over a monolayer of cells that are firmly attached to a surface, usually a glass surface to allow visualization. As an example of this, microfluidic chambers allow the controlled delivery of nanoparticles suspended in fluids over the cell surface, making it possible to quantify the dynamics of polymer particle association with the cultured cells [7].

Migration

The migration of individual cells within a tissue is a critical element in the formation of the architecture of organs and organisms. Similarly, cell migration is likely to be an important phenomenon in tissue engineering, since the ability of cells to move, either in association with the surface of a material or through an ensemble of other cells, will be an essential part of new tissue formation or regeneration. Cell migration is also difficult to measure, particularly in complex environments. Fortunately, a number of useful techniques for quantifying cell migration in certain situations have been developed. As in cell adhesion, however, no technique has gained general acceptance, so it is difficult to correlate results obtained by different techniques or different investigators.

Experimental methods for characterizing cell motility can be divided into visual assays and population assays: a more complete description with early references is available in Chapter 7 of Ref. [3]. In visual assays the movements of a small number (usually $\sim$100) of cells are observed individually. Population techniques, on the other hand, allow the observation of the collective movements of larger numbers of cells: in filter chamber assays, the number of cells migrating through a membrane or filter is measured, while in under-agarose assays, the leading front of cell movement on a surface under a block of agarose is monitored. Both visual and population assays can be quantitatively analyzed, enabling the estimation of

intrinsic cell motility parameters, such as the random motility coefficient and the persistence time: these methods are described in Ref. [3].

Aggregation

Cell aggregates are important tools in the study of tissue development, permitting correlation of cell–cell interactions with cell differentiation, viability and migration, as well as subsequent tissue formation. The aggregate morphology permits reestablishment of cell–cell contacts normally present in tissues; therefore, cell function and survival are often enhanced in aggregate culture. Because of this, cell aggregates may also be useful in tissue engineering, enhancing the function of cell-based hybrid artificial organs or reconstituted tissue transplants [8].

Gentle rotational stirring of suspensions of dispersed cells is the most common method for making cell aggregates [9]. While this method is suitable for aggregation of many cells, serum or serum proteins must be added to promote cell aggregation in many cases, thus making it difficult to characterize the aggregation process and to control the size and composition of the aggregate. Specialized techniques can be used to produce aggregates in certain cases, principally by controlling cell detachment from a solid substratum. For example, stationary culture of hepatocytes above a nonadherent surface [10] or attached to a temperature-sensitive polymer substratum [11] has been used to form aggregates. Synthetic polymers produced by linking cell-binding peptides (such as RGD and YIGSR) to both ends of poly(ethylene glycol) (PEG) have been used to promote aggregation of cells in suspension [12].

The kinetics and extent of aggregation can be measured by a variety of techniques. Often, direct visualization of aggregate size is used to determine the extent of aggregation, following the pioneering work of Moscona [9]. The kinetics of aggregation can be monitored in this manner as well, by measuring aggregate size distributions over time. This procedure is facilitated by the use of computer image analysis techniques or electronic particle counters, where sometimes the disappearance of single cells (instead of the growth of aggregates) is followed. Specialized aggregometers can provide reproducible and rapid measurements of the rate of aggregation; in one such device, small angle light scattering through rotating sample cuvettes is used to produce continuous records of aggregate growth [13].

Cell phenotype

In tissue engineering applications, particularly those in which cell-polymer hybrid materials are prepared, one is usually interested in promotion of some cell-specific function. For example, protein secretion and detoxification are essential functions for hepatocytes used for transplantation or liver support devices; therefore, measurements of protein secretion and intracellular enzyme activity (particularly the hepatic P_{450} enzyme system) are frequently used to assess hepatocyte function. Similarly, the expression and activity of enzymes involved in neurotransmitter metabolism (such as choline acetyltransferase or tyrosine hydroxylase) are often used to assess the function of neurons. Production of extracellular matrix (ECM) proteins is important in the physiology of many tissues, particularly connective tissues and skin; the production of collagen and glycosaminoglycan has been used as an indicator of cell function in chondrocytes, osteoblasts, and fibroblasts. In some cases the important cell function involves the coordinated activity of groups of cells, such as the formation of myotubules in embryonic muscle cell cultures, the contraction of the matrix surrounding the cells, or the coordinated contraction of cardiac muscle cells. In these cases, cell function is measured by the observation of changes in morphology of cultured cells or cell communities.

High-throughput methods for the characterization of cell–polymer interactions

Cell–polymer interactions can be analyzed using methods that allow for many parallel measurements. For example, small spots of ECM proteins can be deposited onto glass slides—or other substrates—to allow for high-throughput screening of cell interactions with ECM proteins and protein combinations [14,15]. This microprinting method can also be applied to three-dimensional (3D) materials appropriate for tissue engineering [16]. Microarray printing of polymer and polymer blends allows for screening of interactions of cells with synthetic polymer surfaces, as well [17]. Although these methods allow for the parallel testing of many different cell/polymer combinations, the measurements of cell interaction are usually limited to light or fluorescence microscopy.

In vivo methods

The context of cell–polymer interactions in vivo is inherently complex due to the presence of blood, interstitial fluids, and the presence of multiple cell types in various activations states. Proteins from the blood and interstitial fluid adsorb to polymer surfaces in a nonspecific manner and provide a substrate for cell adhesion [18,19]. Thus it is believed that cells interact directly with this proteinaceous layer instead of the polymer surface, an assumption that explains the similar in vivo reactions elicited by a diverse array of polymers. Almost all implanted polymers induce a unique inflammatory response termed the foreign body response (FBR) [20,21].

The FBR can be divided in several overlapping phases that include nonspecific protein adsorption, inflammatory cell recruitment—predominantly of neutrophils and macrophages, macrophage fusion to form foreign body giant cells (FBGC), and involvement of fibroblasts and endothelial cells (Fig. 16.1). The end result of the FBR is the formation of FBGC directly on the polymer surface and the subsequent encapsulation of the implant by a fibrous capsule that is largely avascular. To date, the molecular and cellular determinants of the FBR remain largely unknown. However, recent studies have suggested specific roles for several proteins and a key role for macrophages in the FBR. It is now appreciated that the acquisition of specific activation states by these cells is a critical determinant of the FBR, and investigators have sought to influence this process to improve outcomes [22,23].

A number of implantation techniques in rodents and larger animals (typically rabbits, pigs, or sheep) have been adopted for the investigation of cell−polymer interactions. Most notably, short-term studies for the analysis of protein adsorption, inflammatory cell recruitment and adhesion, and macrophage fusion most often employ either intraperitoneal (IP) implantation or the subcutaneous (SC) cage-implant, also known as the wound chamber model. In the IP implant model a sterilized piece of polymer is placed in the peritoneal cavity through a surgical incision [24]. This model has been used extensively in the investigation of protein adsorption onto polymer surfaces and its impact during the early inflammatory response (0−72 hours). In general, due to the short duration, it is difficult to extrapolate the information obtained from such studies to the fate of the FBR. Implant studies of intermediate duration (days to weeks) have been executed using an SC cage-implant; in this system, a polymer is placed within a cage made of stainless steel wire mesh and then implanted through a surgical incision [25]. The SC cage-implant is useful because biologically active soluble agents can be injected through the wire mesh in the immediate vicinity of the polymer.

Both IP and SC implantations allow for the analysis of recruited, nonadherent cell populations, which are collected by lavage of the peritoneal cavity or aspiration of host-derived exudates from the cage. In addition, implant materials can be recovered and analyzed for the presence of adherent cells by various techniques, immunohistochemistry being the most reliable. Thus both implantation models can provide information regarding the migration of cells to the implantation site and the interaction of cells (adhesion, survival, and fusion) with the polymer surface. Other commonly used in vivo models for the analysis of cell−polymer interactions involve the implantation of test polymers directly into host tissues such as dermis (SC), muscle, bone, and brain. Generally, such studies have been carried for longer incubation periods (weeks to months). Furthermore, polymer-based constructs have been surgically implanted at sites where they are expected to perform biological functions, for example, vascular grafts [26,27], heart valves [28], stents [29], and cardiac patches [30]. Thus in addition to biocompatibility and toxicity, polymers can be evaluated for their role in function of a tissue or organ. The direct implant model can be considered the most representative for most polymer applications and can provide information regarding the FBR, such as inflammation (macrophage accumulation and polarization, FBGC formation),

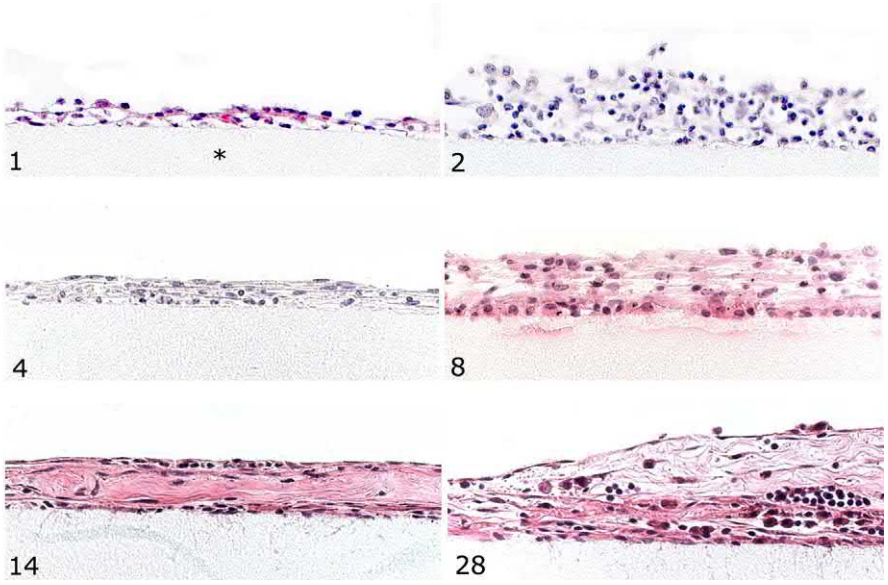


FIGURE 16.1 Development of the foreign body response. Representative images of hematoxylin and eosin−stained Millipore filter disks (mixed cellulose ester) implanted in the peritoneal cavity of wild type mice. The number on each panel indicates the number of days after implantation. In the first 2 days following implantation, the disks display loose and expanding layers of attached inflammatory cells. Between 4 and 8 days, the cell layers show increased organization that appears to coincide with the deposition of collagen fibers. Continuous deposition of collagenous matrix and an overall reduction in cellular content are characteristic of the 2- and 4-week time points. Original magnification 400 ×. * indicates the disk.

fibrosis (collagen content), encapsulation (capsule thickness), and angiogenesis (vascular density). Finally, in cases where the polymer is susceptible to degradation, additional analyses can be performed to evaluate the role of chemical and physical changes of the material in interactions with the tissue.

Cell interactions with polymers

Protein adsorption to polymers

A polymeric material that is placed in solution or implanted in the body becomes coated with proteins quickly, usually within minutes. Many of the subsequent interactions of cells with the material depend on, or derive from, the composition of the protein layer that forms on the surface. Polymers have been shown to adsorb a large number of proteins in vitro [31]. For example, proteomic analysis of polypropylene (PP), polyethylene terephthalate (PET), and polydimethylsiloxane (PDMS) incubated with serum identified immunoglobulins, transferrin, albumin, serum amyloid P, and complement C4, among other proteins, on the polymer surface [32]. The C3 component of complement has also been shown to adsorb to medical grade polyurethanes (PEU) and adhesion of inflammatory cells to the polymer was reduced in the presence of C3-depleted serum but not fibronectin-depleted serum [33]. In contrast, in vivo studies employing the IP implantation method suggest that adsorption of fibrinogen is the critical determinant of the FBR [34]. Specifically, adsorption and denaturation of fibrinogen on polymer surfaces might lead to the exposure of cryptic cell adhesion motifs (integrin-binding sites) that influence subsequent cellular interactions. Despite the recognized importance of adsorbed proteins, it is possible that direct contact between polymers and cells can have significant impact on cell function. Supporting evidence for such interactions was shown in study where large poly(methyl methacrylate) beads (150 μm in diameter) induced the production of a proinflammatory cytokine by macrophages in the absence of serum proteins [35].

Effect of polymer chemistry on cell behavior

Synthetic polymers

For cells attached to a solid substrate, cell behavior and function depend on the characteristics of the substrate. Consider, for example, experiments described by Folkman and Moscona, in which cells were allowed to settle onto surfaces formed by coating conventional tissue-culture PS (TCPS) with various dilutions of poly(2-hydroxyethylmethacrylate) (pHEMA) [36]. As the amount of pHEMA added to the surface was increased, cell spreading decreased as reflected by the average cell height on the surface. The degree of spreading, or average

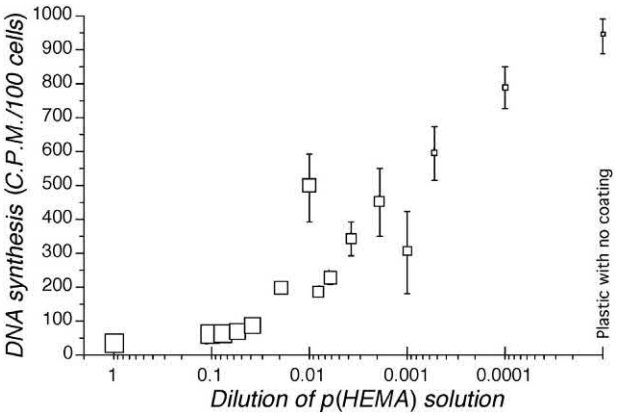


FIGURE 16.2 Cell shape and growth are modulated by properties of a polymer surface. Cell culture surfaces were produced by evaporating diluted solutions of pHEMA onto TCPS. The uptake of [^{3}H]thymidine was used as a measure of proliferation. The size of the symbol represents the relative cell height; small symbols represent cells with small heights, and therefore significant spreading, large symbols represent cells with large heights, and therefore negligible spreading. *pHEM*, Poly(2-hydroxyethylmethacrylate); *TCPS*, tissue-culture polystyrene. *Replotted from Folkman J, Moscona A. Role of cell shape in growth control. Nature 1978;273:345–9.*

height, correlated with the rate of cell growth (Fig. 16.2), suggesting that cell shape, which was determined by the adhesiveness of the surface, modulated cell proliferation. In these experiments, two well-known polymers (TCPS and pHEMA) were used to produce a series of surfaces with graded adhesivity, permitting the identification of an important aspect of cell physiology. These experiments clearly demonstrate that the nature of a polymer surface will have important consequences for cell function, an observation of considerable significance with regard to the use of polymers in tissue engineering.

Following an experimental design similar to that employed by Folkman and Moscona, a number of groups have examined the relationship between chemical or physical characteristics of the substrate and behavior or function of attached cells. For example, in a study of cell adhesion, growth, and collagen synthesis on synthetic polymers, fetal fibroblasts from rat skin were seeded onto surfaces of 13 different polymeric materials [37]. The polymer surfaces had a range of surface energies, as determined by static water contact angles, from very hydrophilic to very hydrophobic. On a few of the surfaces [poly(vinyl alcohol) (PVA) and cellulose], little cell adhesion and no cell growth was observed. On most the remaining surfaces, however, a moderate fraction of the cells adhered to the surface and proliferated. The rate of proliferation was relatively insensitive to surface chemistry: the cell doubling time is ~24 hours, with slightly slower growth observed for two very hydrophobic surfaces [poly(tetrafluoroethylene) (PTFE) and PP]. Collagen biosynthesis was also correlated with contact angle, with

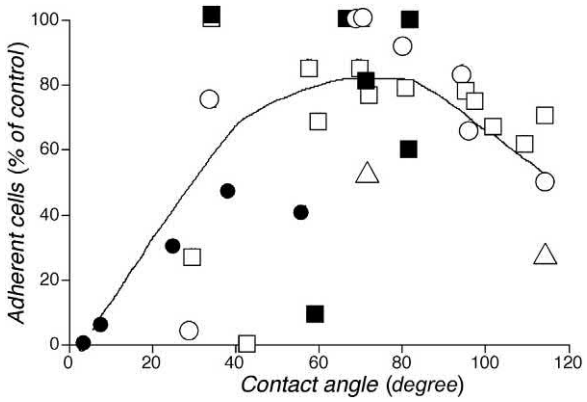


FIGURE 16.3 The relationship between cell adhesion and water-in-air contact angle: fibroblasts (*open squares*), L cells (*open circles*), endothelial cells (*open triangles*), and endothelial cells (*filled circles*). *Replotted from (fibroblasts) Tamada Y, Ikada Y. Fibroblast growth on polymer surfaces and biosynthesis of collagen. J Biomed Mater Res 1994;28:783–9 [37], (L cells) Ikada Y. Surface modification of polymers for medical applications. Biomaterials 1994;15(10):725–36 [38], (endothelial cells) Hasson J, Wiebe D, Abbott W. Adult human vascular endothelial cell attachment and migration on novel bioabsorbable polymers. Arch Surg 1987;122:428–30 [39], (endothelial cells) van Wachem PB, Hogt AH, Beugeling T, Feijen J, Bantjes A, Detmers JP, et al. Adhesion of cultured human endothelial cells onto methacrylate polymers with varying surface wettability and charge. Biomaterials 1987;8:323–8 [40].*

higher rates of collagen synthesis per cell for the most hydrophobic surfaces.

Results from a number of similar studies have been summarized previously, see Tables 12.1 and 12.2 in Ref. [3], for example. Some general conclusions emerge from this past work. For example, in a number of studies, cell adhesion appears to be maximized on surfaces with intermediate wettability (Fig. 16.3). For most surfaces, adhesion requires the presence of serum and, therefore, this optimum is likely related to the ability of proteins to adsorb to the surface. (In the absence of serum, however, adhesion is enhanced on positively charged surfaces, due to the net negative charge of most cell membranes.) Cell spreading on the copolymers of HEMA (hydrophilic) and EMA (hydrophobic) was highest at an intermediate HEMA content, again corresponding to intermediate wettability [41]; in this case, spreading correlated with fibronectin adsorption [41]. The rate of fibroblast growth on polymer surfaces appears to be relatively independent of surface chemistry. Cell viability may also be related to interactions with the surface, although this is not yet predictable. The migration of surface-attached fibroblasts, endothelial cells, and corneal epithelial cells is also a function of polymer surface chemistry, see Ref. [3] for more details.

Surface modification

Polymers can frequently be made more suitable for cell attachment and growth by surface modification. In fact, TCPS substrates used for tissue culture are obtained by the surface treatment of PS by glow discharge or exposure to chemicals, such as sulfuric acid, to increase the number of charged groups at the surface, which improves attachment and growth of many types of cells. Other polymers can also be modified in this manner. Treatment of pHEMA with sulfuric acid, for example, improves adhesion of endothelial cells and permits cell proliferation on the surface [42]. Modification of PS or PET by radio-frequency plasma deposition enhances attachment and spreading of fibroblasts and myoblasts [43]. The effects of these surface modifications appear to be secondary to increased adsorption of cell attachment proteins, such as fibronectin and vitronectin, to the surface. On the other hand, some reports have identified specific chemical groups at the polymer surface—such as hydroxyl (−OH) [44] or surface C−O functionalities [43]—as important factors in modulating the fate of surface-attached cells.

So far, no general principles that would allow prediction of the extent of attachment, spreading, or growth of cultured cells on different polymer surfaces have been identified. Correlations have been made with parameters, such as the density of surface hydroxyl groups [44], density of surface sulfonic groups [45], surface free energy [46,47], fibronectin adsorption [43], and equilibrium water content [48] for specific cells, but exceptions to these correlations are always found. Perhaps general predictive correlations will emerge, as more complete characterization of polymer systems—including bulk properties, surface chemical properties, and nanoscale topography (as described later)—are collected. But many commonly used polymer materials are complicated mixtures, containing components that are added to enhance polymerization or to impart desired physical properties, often in trace quantities. Lot-to-lot variations in the properties of commercially available polymers can be significant.

The surface chemistry of polymers appears to influence cell interactions in vivo. For example, the ability of macrophages to form multinucleated giant cells at the material surface correlates with the presence of certain chemical groups at the surface of hydrogels: macrophage fusion decreases in the order $(CH_3)_2N- > -OH = -CO-NH- > -SO_3H > -COOH$ $(-COONa)$ [49]. A similar hierarchy has been observed for CHO (Chinese Hamster Ovary) cell adhesion and growth on surfaces with grafted functional groups: CHO cell attachment and growth decreased in the order $-CH_2NH_2 > -CH_2OH > -CONH_2 > -COOH$ [50].

Layer-by-layer (LbL) assembly of nanofilms is a versatile approach for the control of surface properties to modulate cell attachment and function. In the most general technique, alternating layers of two polymers—one polycationic and the other polyanionic—are deposited onto a substrate to create a film of controlled thickness

and chemical properties. By using a cell attachment protein, such as fibronectin, as one of the polyelectrolytes, thin films that control cell adhesion to the substrate can be created [51]. Control of conditions during LbL assembly—such as solution pH—can create films with different physical structures, which further modulate cell interactions [52]. Covalent reactions between the polymers—produced either by addition of cross-linking agents after LbL film formation or during the assembly of the film using reactive polymers—allow for the creation of more stable films [53].

Biodegradable polymers

Biodegradable polymers slowly degrade and then dissolve following implantation. This feature may be important for many tissue engineering applications, since the polymer will disappear as functional tissue regenerates. For this reason, interactions of cells with a variety of biodegradable polymers have been studied. Biodegradable polymers may provide an additional level of control over cell interactions: during polymer degradation, the surface of the polymer is constantly renewed, providing a dynamic substrate for cell attachment and growth.

Homopolymers and copolymers of lactic and glycolic acid [poly(L-lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactide-*co*-glycolide) (PLGA)] have been frequently examined as cell culture substrates, since they have been used as implanted sutures for several decades. Many types of cells will attach and grow on these materials. For example, chondrocytes proliferate and secrete glycosaminoglycans within porous meshes of PGA and foams of PLA [54]. Similarly, rat hepatocytes attach to blends of biodegradable PLGA polymers and secrete albumin when maintained in culture [55]. Neonatal rat osteoblasts also attach to PLA, PGA, and PLGA substrates and synthesize collagen in culture [56].

Cell adhesion and function has been examined on materials made from other biodegradable polymers. As an example, cells from an osteogenic cell line attach onto polyphosphazenes produced with a variety of side groups; the rate of cell growth as well as the rate of polymer degradation depends on side group chemistry [57].

The use of degradable polymers increases the complexity of analysis in the in vivo setting because their degradation products can cause excessive and prolonged biological responses. Depending on the material used, degradation products can be released by hydrolysis, enzymatic digestion, degradative activity of macrophages and FBGC, or combinations of these mechanisms. The effects of polymer-derived products that are released by hydrolysis or enzymatic digestion depend on the chemical properties of the polymer and are not difficult to determine. On the other hand the effects of products released due to cellular activities are difficult to predict, because the chemical and biological mechanisms responsible for their generation and release have not been established. Furthermore, the activity of cells, and thus the rate of release, can be influenced by several parameters such as local concentrations of growth factors. It is expected that biodegradable polymers should allow for the favorable resolution of the FBR after their complete disappearance from the host tissue. The disappearance of FBGC from implantation sites after degradation of polymers to microparticles smaller than 10 μm in size has been observed (Kyriakides et al., unpublished data). Furthermore, macrophages were observed to persist until the degradation of the polymer was complete. In SC implantation sites the formation of a vascularized collagenous work as a replacement for the polymer was observed. Presumably, the physiology of the implantation site, the acuteness of the inflammatory response, and the rate of polymer degradation all influence the nature of the FBR. Favorable in vivo results have been obtained with biodegradable elastomeric polyesters composed of poly(diol citrates) [58]. However, the duration of the FBR was shown to exceed 12 months in implanted compressed naltrexone-poly[*trans*-3,6-dimethyl-1,4-dioxane-2,5-dione] (DL-lactide) loaded microspheres [59].

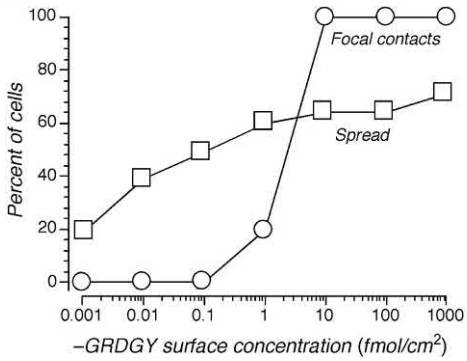
In a direct application the combination of the biodegradable polymers PGA and poly(ε-caprolactone-*co*-L-lactide) has been used successfully as a vascular conduit in humans [60]. Replication of this approach in a rodent model has provided significant insights into cell−polymer interactions and their contribution to vascular remodeling [27]. Specifically, the process was shown to be dependant on the delicate balance between polymer degradation and native ECM deposition [61].

Synthetic polymers with adsorbed proteins

As mentioned earlier, cell interactions with polymer surfaces appear to be mediated by proteins, adsorbed from the local environment. Since it is difficult to study these effects in situ during cell culture, often the polymer surfaces are pretreated with purified protein solutions. In this way the investigators hope that subsequent cell behavior on the surface will be due to the presence of a stable layer of surface-bound protein. A major problem with this approach is the difficulty in determining whether surface conditions, that is, the density of protein on the surface, change during the period of the experiment.

As described earlier, cell spreading, but not attachment, correlates with fibronectin adsorption to a variety of surfaces. Rates of cell migration on a polymer surface are usually sensitive to the concentration of preadsorbed adhesive proteins [62], and migration can be modified by addition of soluble inhibitors to cell adhesion [63].

(A)



(B)

Peptide	Human foreskin fibroblasts	Human vascular smooth muscle cells	Platelets	Human umbilical vein endothelial cells
–GRGDY	85	93	0	90
–GYIGSRY	79	88	0	84
–GPDSGRY	56	62	0	66
–GREDVY	9	7	0	89
–None	9	10	0	8

FIGURE 16.4 Cell adhesion to surfaces with immobilized peptides. (A) Fibroblast spreading on surfaces with immobilized –GRGDY. (B) Cell-selective surfaces: fraction of cells spread for several immobilized peptides. *Data from Hubbell JA, Massia SP, Desai NP, Drumheller PD. Endothelial cell-selective materials for tissue engineering in the vascular graft via a new receptor. Bio/Technology 1991;9:568–72; Massia SP, Hubbell JA. An RGD spacing of 440 nm is sufficient for integrin aVβ3-mediated fibroblast spreading and 140 nm for focal contact and stress fiber formation. J Cell Biol 1991;114:1089–100 [68,69].*

It appears that the rate of migration is optimal at intermediate substrate adhesiveness, as one would expect from mathematical models of cell migration [64]. In fact, a study showed a clear correlation between adhesiveness and migration for CHO cells [65].

The outgrowth of corneal epithelial cells from explanted rabbit corneal tissue has been used as an indicator of cell attachment and migration on biomaterial surfaces [66]. When corneal cell's outgrowth was measured on 10 different materials that were preadsorbed with fibronectin, outgrowth generally increased with the ability of fibronectin to adsorb to the material. Exceptions to this general trend could be found, suggesting that other factors (perhaps stability of the adsorbed protein layer) are also important.

Hybrid polymers with immobilized functional groups

Surface modification techniques have been used to produce polymers with surface properties that are more suitable for cell attachment [38]. For example, chemical groups can be added to change the wettability of the surface, which often influences cell adhesion (Fig. 16.3), as described earlier. Alternatively, whole proteins such as collagen can be immobilized to the surface, providing the cell with a substrate that more closely resembles the ECM found in tissues. Collagen and other ECM molecules have also been incorporated into hydrogels by either adding the protein to a reaction mixture containing monomers and initiating the radical polymerization, or mixing the protein with polymerized polymer, such as pHEMA, in appropriate solvents. To isolate certain features of ECM molecules, and to produce surfaces that are simpler and easier to characterize, smaller biologically active functional groups have been used to modify surfaces. These biologically active groups can be oligopeptides, saccharides, or glycolipids.

Certain short amino acid sequences, identified by the analysis of active fragments of ECM molecules, appear to bind to receptors on cell surfaces and mediate cell adhesion. For example, the cell-binding domain of fibronectin contains the tripeptide RGD (Arg–Gly–Asp) [67].[1] Cells attach to surfaces containing adsorbed oligopeptides with the RGD sequence and soluble, synthetic peptides containing the RGD sequence reduce the cell-binding activity of fibronectin, demonstrating the importance of this sequence in adhesion of cultured cells. A large number of ECM proteins (fibronectin, collagen, vitronectin, thrombospondin, tenascin, laminin, and entactin) contain the RGD sequence. The sequences YIGSR and IKVAV on the A chain of laminin also have cell-binding activity and appear to mediate adhesion in certain cells.

Because RGD appears to be critical in cell adhesion to ECM, many investigators have examined the addition of this sequence to synthetic polymer substrates. The addition of cell-binding peptides to a polymer can induce cell adhesion to otherwise nonadhesive or weakly adhesive surfaces. Cell spreading and focal contact formation are also modulated by the addition of peptide (Fig. 16.4). Since cells contain cell adhesion receptors that recognize only certain ECM molecules, use of an appropriate cell-binding sequence can lead to cell-selective surfaces,

1. Amino acids are identified by their one-letter abbreviation: A = alanine, R = arginine, N = asparagine, D = aspartic acid, B = asparagine or aspartic acid, C = cysteine, Q = glutamine, E = glutamic acid, Z = glutamine or glutamic acid, G = glycine, H = histidine, I = isoleucine, L = leucine, K = lysine, M = methionine, F = phenylalanine, P = proline, S = serine, T = threonine, W = trypotophan, Y = tyrosine, V = valine.

where the population of the cells that adhere to the polymer is determined by the peptide [68].

The presence of serum proteins attenuates the adhesion activity of peptide-grafted PEU surfaces [70], highlighting a difficulty in using these peptide-grafted materials in vivo. This problem may be overcome, however, through the development of base materials that are biocompatible yet resistant to protein adsorption. One of the most successful approaches for reducing protein adsorption or cell adhesion is to produce a surface rich in PEG. A variety of techniques have been used including surface grafting of PEG, adsorption of PEG-containing copolymers, semiinterpenetrating networks, or immobilization of PEG-star polymers to increase the density of PEG chains at the surface. Alternates to PEG—particularly hyperbranched polyglycerol (HPG)—may provide even better resistance to protein adsorption than PEG [71]. Polymer nanoparticles coated with HPG circulate longer after intravenous injection that identical particles coated with PEG, providing further evidence for the decreased interaction of HPG with proteins [72].

As an alternate approach, matrices can be formed directly from synthetic polypeptides. For example, genes coding the β-sheet of silkworm silk have been combined with genes coding fragments of fibronectin to produce proteins that form very stable matrices with cell adhesion domains (Pronectin F). Synthetic proteins based on peptide sequences from elastin have been used as cell culture substrates: in the presence of serum, fibroblasts and endothelial cells adhered to the surfaces of matrices formed by γ-irradiation cross-linking of polypeptides containing repeated sequences GGAP, GGVP, GGIP, and GVGVP [73].

Surface adsorption of homopolymers of basic amino acids, such as polylysine and polyornithine, are frequently used to enhance cell adhesion and growth on polymer surfaces. Similarly, covalently bound amine groups can also influence cell attachment and growth. Polymerization of styrene with monoamine or diamine containing monomers produced copolymers with ∼8% mono- or diamine side chains, which enhanced spreading and growth: diamine-PS > monoamine-PS > PS [74].

The immobilization of saccharide units to polymers can also influence cell attachment and function. As an example, *N*-*p*-vinylbenzyl-*o*-β-D-galactopyranosyl-(1−4)-D-gluconamide has been polymerized to form a polymer with a PS backbone and pendant lactose functionalities [75]. Rat hepatocytes adhere to surfaces formed from this polymer, via asialoglycoprotein receptors on the cell surface, and remain in a rounded morphology consistent with enhanced function in culture. In the absence of serum, rat heptaocytes will adhere to similar polymers with pendant glucose, maltose, or maltotriose. Similar results have been obtained with polymer surfaces derivatized with *N*-acetyl glucosamine, which is recognized by a surface lectin on chicken hepatocytes [76].

Films produced by LbL techniques can also be surface modified to modulate cell interactions. For example, for films that are assembled using covalent reactions, residual reactive groups can be used to attach small molecules that control the interaction of the film with cells. In one study, PEI films produced by covalent LbL assembly were further modified by coupling of small molecules. Coupling of hydrophobic molecules produced films that permitted cell attachment and growth, whereas covalent coupling of hydrophilic sugar groups reduced cell attachment, even in the presence of serum proteins [77].

Electrically charged or electrically conducting polymers

A few studies have examined cell growth and function on polymers that are electrically charged. Piezoelectric polymer films, which were produced by high-intensity corona poling of poly(vinylidene fluoride) or poly(vinylidene fluoride-*co*-trifluoroethylene) and should generate transient surface charge in response to mechanical forces, enhanced the attachment and differentiation of mouse neuroblastoma cells (Nb2a), as determined by neurite number and mean neurite length [78]. These observations may be important in vivo, as well. For example, positively poled poly(vinylidene fluoride-*co*-trifluoroethylene) nerve guidance channels produced greater numbers of myelinated axons than either negatively poled or unpoled channels [79]. Electrically conducting polymers might be useful for tissue engineering applications, because their surface properties can be changed by application of an applied potential. For example, endothelial cells attached and spread on fibronectin-coated polypyrrole films in the oxidized state, but they became rounded and ceased DNA synthesis when the surface was electrically reduced [80].

Influence of surface morphology on cell behavior

The microscale texture of an implanted material can have a significant effect on the behavior of cells in the region of the implant. This has long been observed in vivo. For example, fibrosarcomas developed with high frequency, approaching 50% in certain situations, around implanted Millipore filters; the tumor incidence increased with decreasing pore size in the range of 450−50 μm [81].

More recently, silicone breast implants have been associated with various complications including the development of breast implant−associated anaplastic large cell lymphoma. Epidemiological studies show that the disease

occurs at a much higher frequency in patients with textured versus smooth implants [82].

The behavior of cultured cells on surfaces with edges and grooves is different than behavior on smooth surfaces. In many cases, cells oriented and migrated along fibers or ridges in the surface, a phenomenon called contact guidance from early studies on neuronal cell cultures [83]. Fibroblasts orient on grooved surfaces [84], with the degree of cell orientation depending on both the depth and pitch of the grooves. Not all cells exhibit the same degree of contact guidance when cultured on identical surfaces: a comparison of cell responses to surfaces with grooves and edges, collected from early studies, is provided in Table 12.3 of Ref. [3]. These measurements are relevant to design of materials for tissue engineering: adhesion and initial migration of an osteoblast cell line was examined on materials with grooves ranging in pitch from 150 to 1000 nm and found to be optimal on grooves of intermediate spacing [85]. Cultured stem cells behave differently on grooved surfaces, when compared to smooth surfaces, which appears to be secondary to changes in integrin assembly, focal contact formation, and cytoskeletal organization [86].

Other textures also influence cell behavior. For example, substrates with peaks and valleys influence the function of attached cells [87]. PDMS surfaces with 2−5 μm texture maximized macrophage spreading. Similarly, PDMS surfaces with 4 or 25 μm^2 peaks uniformly distributed on the surface provided better fibroblast growth than 100 μm^2 peaks or 4, 25, or 100 μm^2 valleys.

The microscale structure of a surface has a significant effect on cell migration, at least for the migration of human neutrophils. In one study, microfabrication technology was used to create regular arrays of micron-size holes (2 μm × 2 μm × 210 nm) on fused quartz and photosensitive polyimide surfaces [88]. The patterned surfaces, which mimicked a structural element of 3D network (i.e., spatially separated mechanical edges), were used to study the effect of substrate microgeometry on neutrophil migration. The edge-to-edge spacing between features was systematically varied from 6 to 14 μm with an increment of 2 μm. The presence of evenly distributed holes at the optimal spacing of 10 μm enhanced migration by a factor of 2 on polyimide, a factor of 2.5 on collagen-coated quartz and a factor of 10 on uncoated quartz. The biphasic dependence on the mechanical edges of neutrophil migration on 2D patterned substrate was strikingly similar to that previously observed during neutrophil migration within 3D networks, suggesting that microfabricated materials provide relevant models of 3D structures with precisely defined physical characteristics. Perhaps more importantly, these results illustrate that the microgeometry of a substrate, when considered separately from adhesion, can play a significant role in cell migration.

Use of patterned surfaces to control cell behavior

A variety of techniques have been used to create patterned surfaces containing cell adhesive and nonadhesive regions. Patterned surfaces are useful for examining fundamental determinants of cell adhesion, growth, and function. For example, individual fibroblasts were attached to adhesive micro-islands of palladium that were patterned onto a nonadhesive pHEMA substrate using microlithographic techniques [89]. By varying the size of the micro-island, the extent of spreading and hence the surface area of the cell was controlled. On small islands ($\sim$500 μm^2) cells attached but did not spread. On larger islands (4000 μm^2), cells spread to the same extent as in unconfined monolayer culture. Cells on large islands proliferate at the same rate as cells in conventional culture, and most cells attached to small islands proliferate at the same rate as suspended cells. For 3T3 cells, however, contact with the surface enhanced proliferation, suggesting that anchorage can stimulate cell division by simple contact with the substrate as well as by increases in spreading.

A number of other studies have employed patterned surfaces in cell culture. Micrometer-scale adhesive islands of self-assembled alkanethiols were created on gold surfaces using a simple stamping procedure [90], which served to confine cell spreading islands. When hepatocytes were attached to these surfaces, larger islands (10,000 μm^2) promoted growth, while smaller islands (1600 μm^2) promoted albumin secretion. Stripes of a monoamine-derivatized surface were produced on fluorinated ethylene propylene films by radio-frequency glow discharge [91]. Since proteins adsorbed differently to the monoamine-derivatized and the untreated stripes, striped patterns of cell attachment were produced. A similar approach, using photolithography to produce hydrophilic patterns on a hydrophobic surface, produced complex patterns of neuroblastoma attachment and neurite extension [92]. A variety of substrate microgeometries were created by photochemical fixation of hydrophilic polymers onto TCPS or hydrophobic polymers onto PVA through patterned photomasks: bovine endothelial cells attached and proliferated preferentially on either the TCPS surface (on TCPS/hydrophilic patterns) or the hydrophobic surface (on PVA/hydrophobic patterns) [93]. When chemically patterned substrates were produced on self-assembled monolayer films using microlithographic techniques, neuroblastoma cells attached to and remained confined within amine-rich patterns on these substrates [94].

Cell interactions with polymers in suspension

Most of the studies reviewed in the preceding section concerned the growth, migration, and function of cells attached to a solid polymer surface. This is a relevant paradigm for a variety of tissue engineering applications, where polymers will be used as substrates for the transplantation of cells or as scaffolds to guide tissue regeneration in situ. Polymers may be important in other aspects of tissue engineering, as well. For example, polymer microcarriers can serve as substrates for the suspension culture of anchorage-dependent cells and, therefore, might be valuable for the in vitro expansion of cells or cell transplantation [95]. In addition, immunoprotection of cells suspended within semipermeable polymer membranes is another important approach in tissue engineering, since these encapsulated cells may secrete locally active proteins or function as small endocrine organs within the body.

The idea of using polymer microspheres as particulate carriers for the suspension culture of anchorage-dependent cells was introduced by van Wezel [96]. As described earlier for planar polymer surfaces, the surface characteristics of microcarriers influence cell attachment, growth, and function. In the earliest studies, microspheres composed of diethylaminoethyl (DEAE)-dextran were used; these spheres have a positively charged surface and are routinely used as anion-exchange resins. DEAE-dextran microcarriers support the attachment and growth of both primary cells and cell lines, particularly when the surface charge is optimized. In addition to dextran-based microcarriers, microspheres that support cell attachment can be produced from PS, gelatin, and many of the synthetic and naturally occuring polymers described in the preceding sections. The surface of the microcarrier can be modified chemically, or by immobilization of proteins, peptides, or carbohydrates.

Suspension culture techniques can be used to permit cell interactions with complex 3D polymer formulations, as well. For example, cells seeding onto polymer fiber meshes during suspension culture often results in more uniform cell distribution within the mesh than can be obtained by inoculation in static culture [97].

In cell encapsulation techniques, cells are suspended within thin-walled capsules or solid matrices of polymer. Alginate forms a gel with the addition of divalent cations under very gentle conditions and, therefore, has been frequently used for cell encapsulation. Certain synthetic polymers, such as polyphosphazenes, can also be used to encapsulate cells by cation-induced gelation. Low-melting-temperature agarose has also been studied extensively for cell capsulation. Methods for the microencapsulation of cells within hydrophilic or hydrophobic polyacrylates by interfacial precipitation have been described [98], although the thickness of the capsule can limit the permeation of compounds, including oxygen, through the semipermeable membrane shell. Interfacial polymerization can be used to produce conformal membranes on cells or cell clusters [99], thereby providing immunoprotection while reducing diffusional distances.

Hollow fibers are frequently used for macrocapsulation; cells and cell aggregates are suspended within thin fibers composed of a porous, semipermeable polymer. Chromaffin cells suspended within hollow fibers formed from the copolymer of vinyl chloride and acrylonitrile, which are commonly used as ultrafiltration membranes, have been studied as potential treatments for cancer patients with pain [100], Alzheimer's disease [101], and retinitis pigmentosa [102]. Other polymer materials—such as chitosan, alginate, and agar—have been added to the interior of the hollow fibers to provide an internal matrix that enhances cell function or growth.

Polymer nanoparticles are rapidly becoming important materials for drug delivery and tissue engineering, but little is known about how particles with diameters in the range of 50−200 nm interact with cells. When PLGA nanoparticles ($\sim$100 nm diameter) were suspended over monolayers of airway, intestinal, or renal tubular epithelial cells, particles were internalized as soon as 30 minutes after first exposure, becoming widely distributed through the intracellular space over a 24 hour period [103], but the extent of nanoparticle internalization varied among the epithelial cell types. PS nanoparticle interactions with model cell membranes depend on particle size, shape, and surface chemistry, as well as membrane composition, which may be a factor in the variable interactions of particles with different cells [104]. Although multiple mechanisms of nanoparticle entry into cells have been suggested, endocytosis is important in most cells, although it is not the only mechanism, as shown with endothelial cells [105]. In another example, PS particles (20 nm diameter) translocated—in the apical to basolateral direction—through monolayers of cultured alveolar cells, with particles carrying a positive surface charge moving more effectively than particles with a negative surface charge, apparently by transcellular, nonendocytic mechanisms [106].

Proteins absorb readily to the surface of polymer nanoparticles, as they do to all polymer surfaces. The presence of surface-bound proteins influences the interaction of nanoparticles with cells [107]. Particle shape may also have an influence on polymer particle interactions with cells [108], although there are few studies that have examined the effect of shape, particularly for particles with diameters less than 1 μm. As in other systems, tethering of ligands to the polymer surface can influence nanoparticle interactions with cells, including enhancing association and internalization [109].

Cell interactions with three-dimensional polymer scaffolds and gels

Cells within tissues encounter a complex chemical and physical environment that is quite different from commonly used cell culture conditions. 3D cell culture methods are frequently used to simulate the chemical and physical environment of tissues. Often, tissue-derived cells cultured in ECM gels will reform multicellular structures that are reminiscent of tissue architecture.

Gels of agarose have also been used for 3D cell culture. Chondrocytes dedifferentiate when cultured as monolayers but reexpress a differentiated phenotype when cultured in agarose gels [110]. When fetal striatal cells are suspended in 3D gels of hydroxylated agarose, ~50% of the cells extended neurites in gels containing between 0.5% and 1.25% agarose, but no cells extended neurites at concentrations above 1.5%. This inhibition of neurite outgrowth correlates with an average pore radius of greater than 150 nm [111]. Neurites produced by PC12 cells within agarose gels, even under optimal conditions, are much shorter and fewer in number than neurites produced in gels composed of ECM molecules [112].

Macroporous hydrogels can also be produced from pHEMA-based materials, using either freeze-thaw or porosigen techniques. These materials, when seeded with chondrocytes, may be useful for cartilage replacement [113]. Similar structures can be produced from PVA by freeze-thaw cross-linking. PEG-based macroporous gels were used as a scaffold for endothelial cells to form microvessel networks in vivo [114]. Although cells adhere poorly to pHEMA, PVA, and PEG materials, adhesion proteins or charged polymers can be added during the formation to encourage cell attachment and growth. Alternatively, water-soluble, nonadhesive polymers containing adhesive peptides, such as RGDS, can be photopolymerized to form a gel matrix around cells (see Ref. [115], for example).

Fiber meshes and foams of PLGA, PLA, and PGA have been used to create 3D environments for cell proliferation and function, and to provide structural scaffolds for tissue regeneration. When cultured on 3D PGA fiber meshes, chondrocytes proliferate, produce both glycosaminoglycans and collagen, and form structures that are histologically similar to cartilage [116]. The internal structure of the material, as well as the physical dimensions of the polymer fiber mesh, influences cell growth rate, with slower growth in thicker meshes. Changing the fluid mechanical forces on the cells during the tissue formation also appears to influence the development of tissue structure.

In addition to fiber meshes, porosity can be introduced into polymer films by phase separation, freeze drying, salt leaching, and a variety of other methods (reviewed in Ref. [117]). It is now possible to make porous, degradable scaffolds with controlled pore architectures and oriented pores [118,119]. Fabrication methods that provide control over the structure at different length scales may be useful in the production of 3D tissue-like structures.

Most methods for producing fiber meshes are limited to producing fibers ~10 μm in diameter, which is much larger than the diameter of natural fibers that occur in the ECM, and also larger than many of the features that are known to be important in orienting or guiding cell activity. Electrospinning techniques can be used to make small diameter fibers and nonwoven meshes of a variety of materials including poly(caprolactone) (PCL), PLA, collagen, and elastin mimetic polymers.

Cell interactions unique to the in vivo setting

While cell interactions with polymers in vitro can be described by examination of cell behaviors—such as adhesion, migration, or gene expression—or the coordinated behavior of cell groups—such as aggregation, cell interactions with polymers in vivo can lead to other responses, involving cells that are recruited to the implantation site and remodeling of the tissue space surrounding, or even within, the polymeric material. Inflammation, the FBR, and angiogenesis are three examples of these more global responses to an implanted material.

There is much still to learn in this area, but it is clear that both the implant material and the physiology of the implant site are important variables. A study describing a relatively simple experiment, in which ePTFE implants were placed in adipose tissue, in SC tissue, or epicardially illustrates the variability of these responses [120]. Moreover, studies in monocyte chemoattractant protein (MCP-1)-null mice have shown that the extent of the FBR was dependant on the implantation site [121,122]. This short section introduces these physiological responses to implanted materials.

Inflammation

The implantation of polymers through surgical incisions means that an initial component of the FBR involves a wound healing−like response and it is reasonable to assume that the early inflammatory response is mediated, at least in part, by wound-derived factors. Analysis of inflammatory cells has been pursued in several implantation models and was shown to involve predominantly neutrophils (early) and monocyte/macrophages (late). In addition, dendritic cells and T cells have been implicated in the response, but their roles are not as clearly defined [123,124]. Subsequent to their recruitment, these cells are believed to utilize adhesion receptors to interact with adsorbed proteins. Studies in mice that lack specific integrins or fibrinogen have provided supporting evidence for

this hypothesis [125–127]. Specifically, short-term (18 hours) IP implantation of PET disks in mice that lack fibrinogen indicated normal recruitment but reduced adhesion of macrophages and neutrophils to the polymer. In the same study, analysis of the response in mice that lack plasminogen indicated no changes in cell adhesion to the polymer despite a reduction in the recruitment of both cell types in the peritoneal cavity. Thus in addition to fibrinogen for adhesion, inflammatory cells can utilize plasminogen for migration/recruitment. Moreover, studies in mice lacking plasma fibronectin displayed altered FBR [128]. More recently, it was shown that polymer–macrophage interactions can lead to activation of the inflammasome leading to the secretion of the proinflammatory cytokine interleukin (IL)-1β [35]. In the same study, it was shown that mice that lack components of the inflammasome mount reduced FBR suggesting that initial interactions between cells and polymers are critical determinants of the FBR. Surprisingly, the chemokine CCL2 (also known as MCP-1) was shown not to be important in monocyte/macrophage recruitment in long-term implants in the subcutis [121]. However, CCL2 was shown to be important for macrophage fusion leading to FBGC formation. Subsequently, it was shown that CCL2 was critical for the recruitment of inflammatory cells in the FBR when implants were placed in the peritoneal cavity of mice [122]. Consistent with the notion that macrophage responses are critical in the FBR, IP implants in CCL2-null mice displayed minimal encapsulation.

FBGC can cause damage to polymer surfaces through their degradative and phagocytic activities and, thus, pose a significant obstacle to the successful application of polymer-based biomaterials and devices. In vivo studies have identified a critical role for IL-4 in the formation of FBGC [25], but the regulation of macrophage fusion is not fully understood [129]. Studies in rodents have enhanced our understanding of this process by implicating tumor necrosis factor and the nuclear factor-kappa B pathway [22]. Moreover, polymer (PCL)-mediated release of the inhibitor BAY-11 resulted in partial attenuation of FBGC formation [130]. On the other hand, several studies have focused on the role of polymer surface chemistry on macrophage function and FBGC formation. For example, analysis of macrophage adhesion, apoptosis, and fusion on hydrophobic (PET and BDEDTC-coated), hydrophilic (PAAm), anionic (PAANa), and cationic (DMAPAAmMel) surfaces implanted in the rat cage-implant model revealed that PAAm and PAANa induced more apoptosis and reduced adhesion and fusion [131].

Fibrosis and angiogenesis

Unlike wound healing, the resolution of the polymer-associated inflammatory response is characterized by the excessive deposition of a highly organized collagenous matrix and a striking paucity of blood vessels [132,133]. The collagenous capsule can vary in thickness but usually exceeds 100 μm, presumably to limit diffusion of small molecules to and from the polymer. The dense and organized nature of the collagen fibers in the capsule could play a role in limiting blood vessel formation. Implantation studies in mice that lack the angiogenesis inhibitor TSP2 indicated that an increase in vascular density in capsules surrounding PDMS disks was associated with significant loosening of the collagenous matrix [134]. However, a direct link between the arrangement of collagen fibers in the capsule and blood vessel formation has not been established. Interestingly, the modification of the PDMS surface from a hydrophobic to a hydrophilic state altered its cell adhesive properties in vitro but did not cause a change in the FBR in vivo [134]. Such observations underscore the significance of in vivo evaluation of cell- and tissue–polymer interactions. Reduced encapsulation of PDMS (silicone rubber) disks and cellulose Millipore filters implanted SC has been reported in mice that lack SPARC (secreted protein, acidic, and rich in cysteine), a matricellular glycoprotein that modulates the interactions of cells with the ECM. Interestingly, mice that lack SPARC and its close homolog, hevin, display diminished vascular density in encapsulated Millipore filters (type HA, mixed cellulose ester) [135]. Taken together, implantation studies in genetically modified mice suggest that members of the matricellular protein group play critical roles in the FBR [133,136]. The process however, can also be influenced by parameters such as polymer special geometry and porosity. Comparison of the FBR elicited by expanded and condensed PTFE showed similar encapsulation but more mature fibrous capsule formation in the latter [137]. In addition, the effect of polymer porosity in the FBR was examined in SC-implanted PTFE membranes in rats where it was shown that the vascular density could be increased in capsules surrounding polymers with a pore size in the range of 5 μm [138]. However, it is unclear whether the same porosity would enhance the vascular density of capsules surrounding other polymers. In fact, Sussman et al. showed that pHEMA scaffolds with 34 μm pores were able to modulate macrophage activation and reduce fibrosis and increase vascularization [139].

More recently, charge distribution on polymers has been shown to attenuate the FBR. For example, poly(carboxybetaine methacrylate) (PCBMA) hydrogels were shown to completely resist encapsulation [140]. Similarly, coating of implantable glucose sensors with a zwitterionic polymer (poly-methacryloyloxyethyl phosphorylcholine) resulted in reduction in noise associated with reduced FBR [141].

Finally, an additional concern with polymer encapsulation is the presence of contractile cells, myofibroblasts,

which can cause contraction of the capsule and misshape or damage polymer implants. For example, silicone-based breast implants have been shown to be susceptible to this phenomenon [142].

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Chapter 17

Polymer scaffold fabrication

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Introduction

A multitude of polymer-processing techniques has been used for the fabrication of tissue engineering scaffolds (Table 17.1), which typically serve as temporary structures in place of extracellular matrix (ECM) for the attachment, proliferation, and differentiation of cells. Traditional processing methods have been sufficient to create scaffolds with interconnected porous architecture for nutrient and waste transport as well as the migration of cells and have often incorporated biochemical cues for tissue development in the form of natural polymers, ceramics, or growth factors. However, these scaffolds are typically uniform in composition, limited in the complexity of physical features, and difficult to scale-up in production. Thus more advanced scaffold fabrication techniques have been needed to accomplish the goal of producing tissue- and patient-specific scaffolds in the fields of tissue engineering and regenerative medicine [2]. To address this need, three-dimensional (3D) printing (3DP) technologies have emerged as a collection of powerful processing methods that can create scaffolds of highly defined physical architecture, with the potential to deposit multiple material/ink compositions in a spatially defined manner at high resolution. 3DP technology thus allows for the creation of more highly biomimetic scaffolds that recapitulate both the physical and biochemical features of native tissue ECM. Bioprinting, in particular, is a set of 3DP strategies in which living cells and/or biologically active molecules are encapsulated within the ink to be printed, differing in practice from the fabrication of scaffolds out of purely acellular materials and then seeding cells post-printing [3–5]. In this approach, bioinks, or the cell-containing materials, are often printed in tandem with cell-free support materials to allow for the creation of mechanically stable scaffolds as well as the introduction of cell patterning [5]. Bioactive compounds, such as growth factors and peptides, are often incorporated within the bioink itself to enable localized differentiation of cells to the desired phenotypes. Generally, the principle of 3DP and bioprinting techniques is that individual layers of the scaffold are deposited one by one according to a digital model generated through computer-aided design (CAD) software such as AutoCAD or SolidWorks. The CAD model itself can be designed by the user to match the size and shape of a tissue defect or even acquired from 3D, patient-specific scans using techniques such as magnetic resonance imaging or computed tomography [6]. When designing the digital model for printing, tissue engineers must take into account and replicate not only the overall morphology of the tissue defect but also the necessary porous architecture for a tissue engineering scaffold, as discussed later [3]. The user must also specify the locations for the deposition of different materials/inks, which can be defined either in the model itself or in the printer software [2].

The overarching goal of utilizing 3DP for tissue engineering is to deposit materials, bioactive factors, and/or living cells with precise layer-by-layer spatial control to create biologically functional 3D structures [6], all of which will be discussed in this chapter. For the sake of ensuring that the printed cells remain viable, these bioink components need to be thoroughly screened for their effects on cell morphology, phenotype, and viability, as cells will respond directly to environmental cues present in the bioink [7,8]. The materials themselves, whether cell-encapsulating or cell-free, must be tunable in mechanical and architectural properties to create scaffolds that match the physical and mechanical properties of native tissue [6]. The processing conditions of the printer also play a large part in final cell viability, and so testing

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TABLE 17.1 Methods of scaffold fabrication.

Technique	Description	Advantages	Disadvantages
Rapid prototyping: 3DP	CAD-modeled deposition of material and/or cells, layer-by-layer stacking to create a 3D scaffold	Wide variety of materials, highly defined porosity and architecture, accurate repeatability, allows incorporation of bioactive molecules and cells	High-throughput manufacturing not available, time consuming process
Fiber bonding	Polymer fibers are heated and fused at points of intersection	Simple procedure, high surface area-to-volume ratio promotes cell attachment	High processing temperatures, limited control over porosity
Electrospinning	Voltage is applied to spin polymer fibers in a nonwoven mesh	Very fine fiber thickness maybe achieved	Limited control of fiber deposition and porosity, poor mechanical integrity
Solvent casting/ particulate leaching	Porogen incorporated into polymer and solidified, porogen is later dissolved/leached	Minimal material needed for procedure, high porosity, allows incorporation of composite materials	Pore structures not interconnected, residual solvents or porogens, detrimental to bioactivity
Melt molding	Polymer and porogen heated in mold, solidification and removal from mold, porogen leached out	Control of shape with mold, flexible geometry, allows incorporation of composite materials	High processing temperatures, lack of pore interconnectivity
Membrane lamination	Thin layers of porous polymer are chemically fused	Defined contours and geometries, no visible boundary between layers	Necessary to define shape for each layer, time consuming, cytotoxic solvents
Extrusion	Polymer is heated and forced through a die, forms 3D profile of die's cross-sectional area	Ability to fabricate tubular structures	High temperatures or pressures needed
Freeze-drying	Polymer solution is emulsified, emulsion poured in mold and frozen	Some pore interconnectivity	Lower porosity than most other procedures, scaffold and pore size limitations
Phase separation	Polymer dissolved in solvent, bioactive molecules added to make homogenous mixture, cooling and freezing separates phases, solvent sublimed	Potential for drug delivery applications, bioactive molecules protected from solvents	Residual solvents, limited pore sizes
High internal-phase emulsion	Water-in-oil emulsion following HIPE standards, monomers from organic phase used to synthesize and cross-link polymer scaffolds with pores	Injectability, good pore morphology, biodegradable polymers maybe used	Limited polymer types, high processing temperatures
Gas foaming	Compressed polymer is exposed to high pressure gas, pores form with decreased pressure	Eliminates need for organic solvents, improved cell adhesion	Lack of pore interconnectivity, limited pore sizes
Peptide self-assembly	Designer peptides form into complex geometries (spheres, fibers, and sheets) via hydrophobic/hydrophilic interactions	Maybe designed to promote various cell behaviors, compatible with in vitro culture	Expensive materials, design expertise required, limited scaffold size
In situ polymerization	Polymer is injected at site and polymerizes or cross-links after implantation	Immediate implantation possible, minimal processing necessary	Limited mechanical properties, limited porosity, harmful polymerization by-products, limited material choice

3DP, 3D printing; *CAD*, computer-aided design; *HIPE*, high internal phase emulsion.
Source: Adapted with permission from Trachtenberg JE, Kasper FK, Mikos AG. Chapter 22 — Polymer scaffold fabrication. In: Lanza R, Langer R, Vacanti J, editors. Principles of tissue engineering, 4th ed. Boston, MA: Academic Press; 2014. p. 423–40 [1].

biocompatibility of a bioink extends beyond studies of the material itself and must also include the dynamic behavior of the bioink during the printing process. Common processes for ensuring cytocompatibility and characterization methods for cell viability and activity will thus be discussed later in this chapter.

Design inputs: materials, processing, and cell types

Creating biomimetic scaffolds using 3DP requires careful consideration of not only the cell-encapsulated and cell-free scaffold materials themselves, but also the processing methods for these materials and the types of cells that are to be encapsulated within the scaffold. For example, not only would the polymer need to be sterile filtered and/or prepared in a sterile environment but also the nozzle, cartridge, and print platform would all need to be sterilized and the printer would also require operation within a sterile environment, such as a biosafety cabinet, since they all interface with live cells that are being handled for culturing, and ideally, later implantation for regenerative medicine and tissue engineering applications. After all these precautions are taken to minimize potentially damaging effects to living cells, the successful preservation of cellular metabolism and differentiation must be characterized in these bioprinted structures [9,10]. The results of investigations into cell morphology or activity of the included biological factors can then be compared to what is considered acceptable for specific cell lineages, to ensure that proper environmental conditions are utilized during printing. This section thus describes the various design parameters of a 3DP scaffold ranging from material selection to processing technique and cell types, as well as the different means of characterizing cell viability and activity within printed scaffolds.

Materials and inks

Designing multilayered scaffolds for 3DP requires the use of specific materials with both mechanical and biological properties that mimic those of the target tissue [11]. These biomaterials can be either synthetic or natural polymers that mimic the various properties of living tissue, ranging from architectural and mechanical features to biochemical cues for tissue development. The selection of materials also depends on the 3DP technique used. Extrusion-based printing often involves heating of the material to its glass transition or melting temperature, so any cells or additional compounds in the ink must be able to withstand the thermal stress, and it may thus be favorable in the case of cell encapsulation to extrude polymers with low glass transition temperatures. Thus material selection involves not only the mimicry of native tissue properties but also the consideration of other parameters such as cell type and processing method.

Synthetic polymers, such as poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL), nylon, polycarbonate, and poly(vinyl alcohol), are among the most commonly used materials in 3DP technology because their tunable properties allow for the formation of highly defined or complex scaffolds [11]. A summary of some of the most commonly printed synthetic polymers and their unique advantages and disadvantages for printing can be found in Table 17.2. Polyesters, especially PLA and PCL, have low melting points that allow for increased printability and processability during the 3DP process. These polymers also have the advantage of undergoing hydrolysis during in vivo conditions, which allows biological excretory pathways to eliminate the polymers and their degradation products over time. Synthetic polymers can also be combined with additives, such as biomolecules or ceramics, to maximize the effectiveness of specific desired properties. For example, combinations, such as PCL/chitosan and PCL/β-tricalcium phosphate (TCP), can increase the cartilage and bone-specific bioactivity, respectively, of the printed scaffold [25]. Synthetic polymers are also advantageous because they are typically biologically inert and allow for minimal unwanted cell interactions after cells are seeded or deposited around them. However, cells cannot be encapsulated in many synthetic polymer inks, such as PCL and PLA, because these synthetic polymers have high melting temperatures that require the extrusion to be performed at temperatures much higher than 40 °C or with the usage of organic solvents, both of which are not viable for cells [26]. To tackle the temperature issue, cells can be printed using a layer-by-layer technique where they are encapsulated in a secondary protein or natural polymer ink that is printed simultaneously at low temperature [11]. Alternatively, some water-soluble synthetic polymers, such as poly(ethylene glycol) (PEG), can be used for cell encapsulation when extruded at low temperature in hydrogel form [9].

Natural polymers and proteins are less tunable than synthetic polymers, but they have greater bioactive properties that provide tissue-specific cues for targeted regions of the scaffold. A few commonly printed natural polymers are summarized in Table 17.2. These natural polymers are typically processed by mild printing conditions and are incompatible with high temperatures and strong organic solvents due to degradation or denaturation of the proteins or polymers [11]. Natural polymers, such as agar/agarose, have been used to print biocompatible hydrogels that are suitable for cell encapsulation [18]. Collagen and other matrix-derived proteins can be used to create biomaterial scaffolds with specific peptide sequences that promote cell−matrix interactions and cell function. Similarly, the protein fibrin, a natural polymer with blood-clotting

TABLE 17.2 Commonly printed synthetic and natural polymers for tissue engineering.

Polymer	Compatible 3DP methods	Properties	References
PCL *Synthetic polymer*	• Stereolithography • LAB • Extrusion	• Exhibits well-established biocompatibility • Contains hydrolysable ester bonds • Enables long lifespan—can safely degrade in body at rate similar to that of new bone formation	[12–14]
PGA, PLA, and PLGA *Synthetic polymer*	• Stereolithography • Extrusion	• Exhibits well-established biocompatibility • Contains hydrolysable ester bonds • Enables shorter lifespan, but tunable depending on comonomer ratio and molecular weight	[12,14,15]
PEG derivatives *Synthetic polymer*	• Stereolithography • Extrusion	• Exhibits well-established biocompatibility • Provides hydrophilic and very bioinert material • Can be covalently cross-linked by various methods	[12,14–16]
PVA *Synthetic polymer*	• LAB	• Provides high mechanical strength • Experiences minimal thermal degradation	[12,17]
Agar/agarose *Natural polymer*	• Extrusion	• Exhibits well-established biocompatibility • Provides low viscosity for printing • Provides large gelling temperature range	[12,18]
Alginate *Natural polymer*	• Extrusion	• Exhibits well-established biocompatibility • Allows for fast ionic cross-linking	[12,16]
Collagen/gelatin *Natural polymer*	• Extrusion • LAB	• Exhibits well-established biocompatibility • Supports cell–matrix interactions • Solubilizes in water at body temperature (gelatin)	[12,19]
Fibrin *Natural polymer*	• Extrusion	• Provides blood-clotting properties • Provides strong tissue adhesion • Possesses low mechanical strength	[12,19]
Hyaluronic acid *Natural polymer*	• Extrusion	• Mimics soft tissue ECM • Provides high viscosity and highly hydrated environment	[9,20–22]
Starch *Natural polymer*	• Inkjet printing	• Forms viscous paste when heated	[23,24]

3DP, 3D printing; *ECM*, extracellular matrix; *LAB*, laser-assisted bioprinting; *PCL*, poly(ε-caprolactone); *PEG*, poly(ethylene glycol); *PGA*, poly(glycolic acid); *PLA*, poly(lactic acid); *PLGA*, poly(lactic-co-glycolic acid); *PVA*, polyvinyl alcohol.

properties for wound repair, has high adhesion properties that can promote both cell attachment and tissue integration [19]. Natural polymers, however, are typically too weak to mimic the natural mechanical environment for harder tissues and will also struggle to maintain the integrity of the 3DP scaffold in harder tissues and load-bearing settings [15]. This limitation also prevents many natural polymers from being used to print architecturally complex structures [15]. People have addressed these weaknesses by cross-linking polymers, such as fibrin and collagen, after printing. Chemical cross-linking with glutaraldehyde or ultraviolet (UV)-induced cross-linking postprinting, for instance, can be used to strengthen many natural polymer scaffolds [27]. Coprinting natural and synthetic polymers has also been used to strengthen natural polymer scaffolds. By printing synthetic polymers, such as poly(α-hydroxyacid)s, with natural polymers, such as collagen and hyaluronic acid, researchers have developed hybrid scaffolds that retain the superior qualities of each component. These hybrid scaffolds have the bioactivity and ECM-like qualities of natural polymers while possessing the higher mechanical strength and tunability of synthetic polymers [15]. Overall, 3DP scaffolds that utilize both natural and synthetic polymer inks have been used to address the unique advantages and drawbacks of each to create scaffolds that maximize mechanical strength and integrity while retaining biocompatibility and bioactivity for the tissue of interest.

When selecting biomaterials for printing, one must also consider the degradation kinetics and breakdown products of the material. The speed of degradation should ideally match the rate of matrix regeneration produced by encapsulated and native cells, and more in-depth discussion of these can be found in other chapters of the book. The biological effects of a bioink polymer's degradation can also be wide-ranging. Polyesters, such as PLA and

PCL, for instance, degrade to acidic byproducts that can decrease the local pH around the scaffold. Thus while the degree of degradation in certain synthetic polymers can be more easily controlled by changing parameters, such as molecular weight, the oligomers and monomers that result from their breakdown have the potential to be inflammatory or cytotoxic [6]. This also means that synthetic polymers must be printed at temperatures low enough that they do not experience thermal degradation, which can result in acidic or otherwise cytotoxic monomers and oligomers being present in the printed fibers. Generally, printing at temperatures slightly above the glass transition or melting points is sufficient to avoid any thermal degradation, while producing a low-viscosity melt is suitable for deposition [28].

Processing and cell viability

The usage of 3DP techniques introduces many new external processing conditions that affect cell viability and function, such as in situ polymerization and cross-linking, process temperature, additional pressure or force during deposition, and shear stress, many of which are not typical considerations for traditional studies that seed cells after printing is complete [20,29–32] (see Fig. 17.1 for a summary of these factors [30]). The cross-linking of a bioink's polymer network can enhance the elastic moduli of printed geometries to the point where they possess sufficient mechanical integrity to be handled without damage, and this increase in the stiffness of the ECM can also induce biomechanical cues that drive differentiation down particular cell lineages [6,33]. Depending on the photoinitiator and polymerization system, additional UV or visible light maybe used in conjunction with successive layer fabrication [6]. Extended exposure to UV light is known to have a cytotoxic effect on cell cultures [29,34–36]. To avoid subjecting the cells to UV light, certain studies have employed the use of "partial cross-linking" before the bioink is extruded so that the solution is self-supporting on the build platform, with full cross-linking completed later with additional exposure to the chosen mechanism, such as UV light or calcium ion chelation, to give the construct its full structural integrity [37–39].

Besides the chemical additives and reactive components of a bioink, the dynamic behavior of the bioink itself as it is displaced from the cartridge to the build platform also impacts cell viability. For example, it is known the acellular polymer viscosity has a substantial impact on the injectability of the solution [26,40]. A more viscous solution requires a greater pressure to achieve extrusion through a fixed nozzle diameter, and this would consequently exert greater shear stress on both the material itself and the encapsulated cells near the walls of the nozzle [37,41]. Thus printing cells within a near-liquid polymer solution is advantageous because of a lower required deposition pressure, but that bioink material must be gelled enough to ensure a homogenous cell distribution, as gravitational forces cause cells to settle to the bottom of suspensions when the material viscosity is low [30,42] Alternatively, if a bioink is composed of shear-thinning materials, the resulting drop in polymer viscosity under shear force can decrease the cytotoxic wall forces throughout an extrusion process and preserve cell viability [43,44]. Laser-assisted and inkjet-based bioprinting methods avoid the same degree of shear stress experienced in extrusion bioprinting due to the lack of ink needing to be

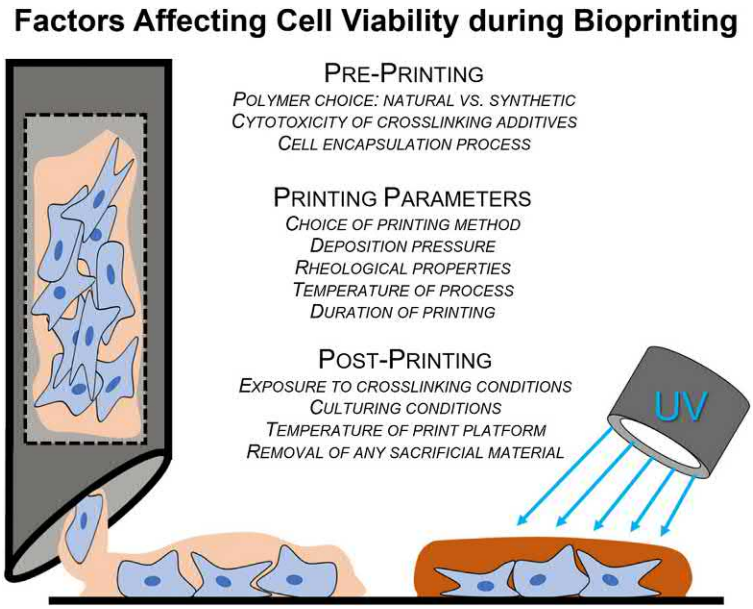

FIGURE 17.1 Preprinting conditions and bioink properties, selection of printing parameters, and postprinting conditions all impact cell viability. These factors can also directly affect subsequent cell proliferation, cell differentiation, and functional tissue formation.

pushed through a nozzle assembly, but these methods have slower processing times and are not able to print with the same cell densities as extruded bioinks [4]. As a result, the biocompatibility of any bioprinting method, especially if it involves shear stress from a printing nozzle or UV irradiation for photo-crosslinking, should always be ensured via thorough characterization of cell viability or comparisons with in vitro control cultures to determine the impact of processing effects [6,9,10,45].

To observe the effect that cross-linking a bioink matrix has on the encapsulated cells' viability, studies typically must first perform rheological analyses to assess the degree of change on the mechanical properties of a material. In photo-crosslinkable bioinks, for example, the effect of varying degrees of cross-linker on resulting shear storage and loss moduli of the raw materials can be measured using a UV-compatible rheometer, where the obtained results would allow for tuning of the balance between printability and desired mechanical stiffness [38,44]. Concurrently, an assessment of the cytotoxicity effect of cross-linking reagent exposure during culturing without the additional factors of applied mechanical forces would identify the upper limit of what can be included in the bioink formulation [9,46]. As discussed in the next section, histological assays, proliferation assays, or fluorescent microscopy utilizing immunofluorescent antibodies that bind to surface markers that indicate the appropriate cell phenotype maybe performed after printing to determine viability and the resulting cell differentiation pathways among cross-linked scaffolds [9,31].

Instead of removing cytotoxic cross-linking mechanisms, some researchers have opted to instead focus on methods that protect cells from these effects during the printing or cross-linking process. One tool commonly employed is the encapsulation of cells in microparticles consisting of cross-linked gelatin, a common natural hydrogel material in which many cell types have been shown to remain viable [2,3,9]. By combining this proven viability with the ability to tune the degradation of the cross-linked polymer network, microparticles allow cells to remain viable during the additional stresses encountered during bioprinting and then are suited to allow for cell migration and proliferation throughout the printed construct via degradation of the network [47–49]. Also, the use of microgels and nanogels, which are aggregates of small polymeric particles in a suspension, creates a viscoelastic environment that is robust enough to be mechanically self-standing after printing, but also has shear-thinning properties that allow it to be extruded without harming encapsulated cells [21]. Supramolecular assembly and guest–host chemistry have been used to create an encapsulating hydrogel material that reversibly binds upon extrusion, allowing for the increased display of cytocompatible shear-thinning properties [20,44].

Alongside in situ polymerization, cross-linking reagents, and damaging shear stress, temperature influences the choice of materials that maybe used for cell-based printing applications. While synthetic polymers have the strength to mimic the mechanical properties needed for supportive tissue applications, such as bone, most of these hydrophobic plastic materials cannot be extruded in liquid form at near-ambient temperatures, as discussed earlier [40,43]. However, since bioink and hydrogel extrusion can be performed at ambient temperature, bioprinted constructs can be fabricated in a normal laboratory setting [37,41,50]. While it is now commonplace in research to combine cells and supportive matrix material during fabrication of living composites, recent investigations have tried combining these separate living and supportive materials through the parallel printing of hydrogel-laden cells and rigid, supportive scaffolds for more robust tissue engineering constructs. The integrated tissue–organ printer (ITOP) is one system that utilizes multiple functional nozzles to print cell-laden hydrogels alongside rigid, biodegradable synthetic polymers in intricate patterns to ultimately fabricate living, human-scale constructs [51].

Cell types and biological interactions

Due to the large variety of environmental factors, processing conditions, and bioink components, researchers must also consider the tolerances of a particular cell type when choosing bioprinting as a method of scientific investigation. Previous studies in the area of bioprinting use cell types that are known to be capable of substantial proliferation and are very robust, such as fibroblasts or transformed cell lines, because they have been shown to tolerate the stresses incurred during construct fabrication, such as UV irradiation and shear stress mentioned in the previous section [6]. As an example of challenges with printing multiple cell types, consider the osteochondral unit. Within this singular structure, there are two supportive biological materials that lie on opposite ends of the spectrum of mechanical properties within the musculoskeletal system: bone and cartilage. While trabecular bone has an elastic modulus on the order of 10 GPa, hyaline cartilage has an elastic modulus on the order of 1 MPa, and so these cells experience markedly different magnitudes of compressive forces in vivo [52–55]. Hyaline cartilage is much softer because its structure consists mainly of water (68%–85%), followed by Type II collagen (10%–20%), and proteoglycans (5%–10%), whereas subchondral bone is predominantly Type I collagen impregnated with nanocrystallite particles of carbonated apatite and forming a much more reinforced composite [54]. Because of the different microenvironments of these cells, subjecting them to forces during printing that are outside

the range of biomechanical stimulation that they naturally experience in vivo may trigger undesirable effects, such as subphysiological or supraphysiological gene expression or cellular hypertrophy, which then causes the printed tissue to be functionally different than the corresponding native tissue [56].

Instead of using mature and fully differentiated cells for cell therapies, the use of stem cells, especially bone marrow−derived human mesenchymal stem cells (MSCs) (hMSCs), has been employed for many bone and cartilage tissue engineering studies [57]. hMSCs are relatively abundant, and if given certain stimulation and biological factors, can differentiate along osteogenic, chondrogenic, or myogenic pathways [58−61]. As discussed in this chapter, bioprinting can allow for the increased control needed for engineering complex tissues, such as positioning stem cells and chondrocytes in stratified layers of a hydrogel for cartilage tissue engineering, but this control should not sacrifice the utility that the undifferentiated stem cells provide [62]. For example, even moderate shear stress was found to have an effect on stem cell differentiation [63]. Therefore in order to maintain the desired undifferentiated state, it is best to use bioprinting methods that reduce the amount of shear stress experienced by cells, such as laser-assisted bioprinting (LAB) and inkjet printing. A well-characterized microvalve-based system has been used to study this safeguard, showing how below a certain threshold of shear stress, the chosen hMSC phenotype is unchanged by the forces experienced during inkjet bioprinting [31]. Within LAB, different conditions for the formation of the microscopic inkjet during cell printing may also be used to ensure high MSC viability in printed cells [64].

While MSCs with osteogenic growth factors are typically the most common cell type used for bioprinting bone tissue, printing of osteoblasts directly has also been employed [57,65,66]. In one study, researchers printed an acellular PCL framework and then seeded the scaffold with osteoblasts and chondrocytes suspended in alginate [66]. The osteoblast cells proliferated, but the chondrocytes did not, despite being viable. One challenge with chondrocytes is that they usually require a supportive matrix for stabilization [57]. In addition, it must be considered that primary chondrocytes are prone to losing their viability and functionality [66]. Therefore bioprinted constructs must be robust enough to be cultured for the extended periods of time necessary for chondrocytes to proliferate before clinical translation is considered.

Another cell−material interaction that is important to consider is the potential for matrix remodeling. This phenomenon is influenced both by the capability of a cell to direct and modify its microenvironment and the suitability of the encapsulating materials for these cell-directed changes. For example, natural polymer hydrogels have a myriad of natural binding sites, biochemical cues, and endogenous factors present, which are known to promote cellular metabolisms closer to that of the native physiology. However, when studying the isolated metabolic pathways or signaling mechanisms involved in this cause-and-effect relationship, these natural polymers are harder to tune due to the diversity in the assortment of confounding factors from ECM cues being presented, as well as the batch-to-batch variability from natural material sourcing [67]. Synthetic polymers typically offer no binding sites and must be modified to possess the biochemical cues that work with native cell remodeling processes [67,68]. The foundation for the mechanisms in these processes includes matrix metalloproteinases (MMPs) and changes in cell-binding domains and integrin expression [69]. Migration of cells is necessary for healthy tissue metabolism as the population grows and proliferates, and what makes this possible is integrin-binding that provides traction for cells to move and penetrate the extracellular environment, which can either be proteolytic, as in the case of main enzymes involved in ECM remodeling, or nonproteolytic [69−71]. This, in order to prevent implant dehiscence or failure in the mechanical properties of implanted cellular composites, the inclusion of MMP-degradable components must be considered at the cellular level and should be tuned to match the remodeling and degradation capabilities of that cell type's biology for the targeted tissue [72,73].

Besides MMP-degradable linkages, polymers can be synthesized or conjugated with certain bioactive factors to tune scaffolds to be more tissue-specific. Whether these are chemical moieties, added cell-adherent peptide sequences, or suspended ceramic materials, bottom-up approaches such as these can be used with both naturally sourced and synthetic polymer materials to control cell−polymer interaction. For example, in osteochondral tissue engineering, printed scaffolds are often designed to be osteoinductive, meaning that they are stimulating for undifferentiated cells toward a bone-forming cell lineage, or osteoconductive, which permits bone tissue to grow on the scaffold surface [74,75]. To both trigger differentiation and subsequent pre-osteoblast activity, scaffolds can be tailored to stimulate these effects of calcium and bone tissue deposition over time through the inclusion of bioceramic materials, such as hydroxyapatite or β-TCP, where cell-directed processes fortify scaffolds to be in the range of stiffness comparable to human bone [72,75−77]. The added complexity and strength of these bioactive, composite scaffolds demonstrate a synergistic effect between stimulated cellular behavior and resemblance of the final scaffold to the target tissue.

Assessment of cell viability and activity

To determine the degree of cytotoxicity or bioactivity of scaffolds and cell-laden constructs, a variety of different

approaches can be employed. Since cell seeding, attachment, spreading, and migration may take place over a period of multiple days, scaffolds are typically assessed for viability within a few hours up to 1 day from fabrication. Here, "viability" is strictly an assessment of whether cells survived the printing process within bioinks or if the scaffold material was cytotoxic. One technique that has been in use for decades is a "live-dead stain" [78,79]. Calcein acetoxymethyl is a molecule that can penetrate cellular membranes, and once cleaved by intracellular esterases of living cells, its products give a strong green fluorescent signal [80,81]. Ethidium homodimer-1 or propidium iodide cannot penetrate cell membranes of living cells, but they are able to bind to the DNA of dead cells with disrupted membranes and give off a strong red fluorescent signal [80,81]. With laser scanning confocal microscopy able to quantify the signals in a target volume, many bioprinting research studies utilize this assay within their printed constructs [21,29,31,32,39,55,64,66,82–89]. To take a closer look at the pathways triggering cellular death, terminal deoxynucleotidyl transferase dUTP nick end labeling staining with fluorescent-activated cell sorting [90] or immunohistochemistry protocols targeting caspase-3 can indicate the degree of apoptotic function in a cell population [91–93]. Another cellular stain, trypan blue, can be used as a cell-excluding stain as it penetrates dead cell membranes and fluoresces, but it is variable and not as greatly utilized [94].

Proliferation and prolonged healthy cell function, which is just as important as cells' immediate state upon going through the 3DP process, is typically assessed in vitro at least 1 day to 1 week after seeding/encapsulation, with some studies extending the culturing period to even 2 or 3 weeks [31,32,39,51,60,66,82,83,86,87,89,95]. Common ways to assess proliferation focus on mitochondrial-based activity in the cell, such as the MTS assay [83]. Other methods look at mitosis, such as methods detecting KI67, which is a protein heavily correlated with proliferation because it is present in all phases of the cell cycle except G0, which is where quiescent cells remain [95–97].

To achieve the goal of engineering cell populations for replacing or regenerating particular living tissues, researchers must subsequently ensure that the cells associated with the fabricated composites are functioning in the desired way that mimics their targeted native activity. There are many ways to assess cellular phenotype, and since both recapitulating anatomic complexity and observing any reorganization of the microenvironment are often of primary concern, many of these techniques rely on different imaging modalities. For example, immunofluorescent staining for expression of certain surface markers can assess if the cellular phenotype matches with that of a certain cell lineage [31,51,95]. This surface-tagged epifluorescence can be combined with 4′,6-diamidino-2-phenylindole or rhodamine phalloidin stains for illustrating cell nuclei and filamentous actin, respectively, to reveal individual cell morphology and the degree of cell spreading on a scaffold or within a matrix [61,83]. Other assays can indicate specific phenotype by confirming a unique aspect of a cell's metabolism, such as alkaline phosphatase activity or looking at mineralization of the scaffold, which are unique to the structural environment created by and necessary for osteoblast proliferation and bone regeneration [56,76,98,99]. Visual qualification through histological analysis can also reveal cell phenotype and any resulting organization of the greater tissue microenvironment in microscale slices of fixed tissue. Common reagents for assessing ECM morphology through immunohistochemistry include hematoxylin and eosin for labeling nuclei and intra/extracellular proteins and membranes; toluidine blue for coloring nuclei and polysaccharides, among others; Masson's trichrome stain for connective tissue studies; Alizarin red S or von Kossa stains for calcium deposition and matrix mineralization; alcian blue or periodic acid–Schiff staining for polysaccharides or carbohydrate-based macromolecules such as those in cartilaginous glycosaminoglycans [51,89,100]. Also, in applications, such as the engineering of cartilage tissue, it is common to apply a Safranin O histological stain when working with chondrocytes to qualify the typically high degree of proteoglycan deposition [56,82].

Scaffolds and cell–matrix composites can be screened for the degree of distribution of both cells or other included noncellular materials, such as bioceramics or microparticles. Scanning electron microscopy (SEM) is a high-resolution method of visually assessing cellular morphology, cell distribution, and spreading, but this method requires more expensive equipment [89]. If a ceramic material is included, such as when hydroxyapatite or β-TCP particles are used to tune degradation for bone tissue engineering applications, micro-computed tomography (μCT) can reveal the degree of dispersion within the printed fibers, whereas SEM would not be able to assess internal properties [72].

3D printing systems and printer types

In 1984 3DP was first patented by Charles Hull for what is now strictly referred to as stereolithography (SLA)-based printing, and 3DP has since evolved into an umbrella term that covers methods of fabrication that build one layer at a time using polymers, metals, or composite materials such as carbon fiber [6,101]. For the modern tissue engineer, there is now an abundance of cutting-edge 3DP technologies at one's disposal for fabricating complex, tissue-specific constructs containing living cells or biological materials. Due to processing

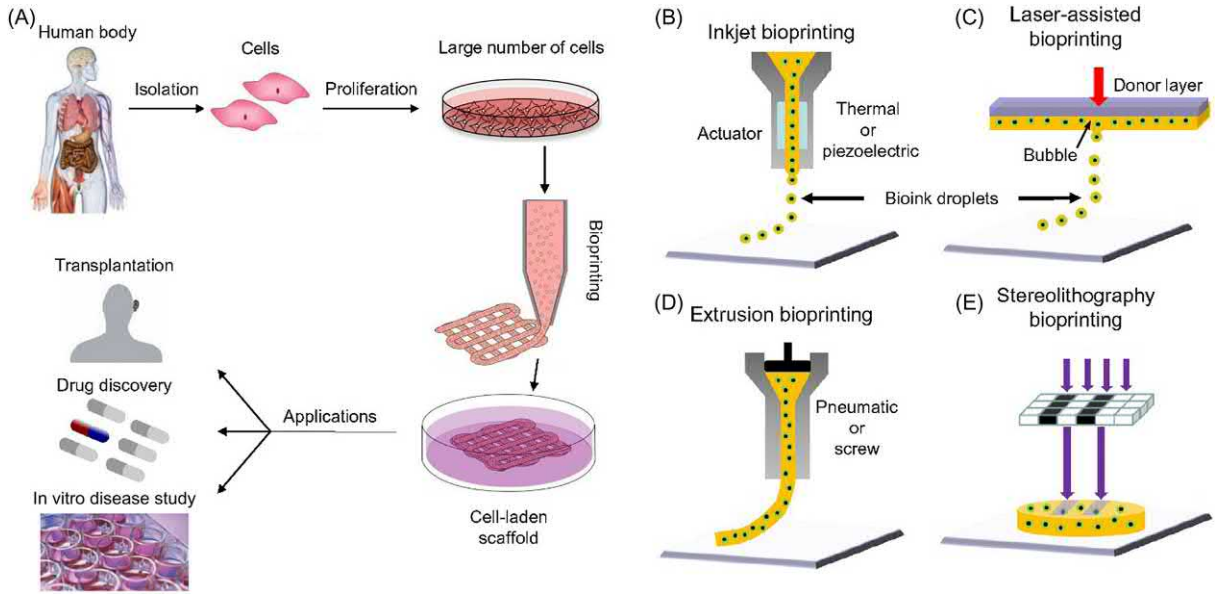

FIGURE 17.2 Bioprinting methods. (A) For human therapeutic applications the typical workflow of bioprinting would involve the isolation and expansion of human cells prior to printing the desired cell-laden scaffold. These scaffolds could then ultimately be used as therapeutic devices themselves, as a testing platform for drug screening and discovery, or as an in vitro model system for disease. (B) Inkjet printers eject small droplets of cells and hydrogel sequentially to build up tissues. (C) Laser bioprinters use a laser to vaporize a region in the donor layer (top) forming a bubble that propels a suspended bioink to fall onto the substrate. (D) Extrusion bioprinters use pneumatic or manual force to continuously extrude a liquid cell−hydrogel solution. (E) Stereolithographic printers use a digital light projector to selectively cross-link bioinks plane by plane. In (C) and (E), colored arrows represent a laser pulse or projected light, respectively. *Reproduced with permission from Mandrycky C, Wang Z, Kim K, Kim DH. 3D bioprinting for engineering complex tissues. Biotechnol Adv 2016;34:422−34.*

considerations mentioned earlier and their effect on cell viability and function, the bioprinting of cell-encapsulated inks is limited to cytocompatible methods, namely, liquid or soft polymer−based modalities [4,22]. The choice of printing modality is also an important one, as the technology-specific limitations discussed later may make one method more suited for recapitulating a certain tissue type than another. The main categories of modern bioprinting methods can be divided into inkjet printing, extrusion, LAB, and SLA (Fig. 17.2). The following section will discuss the advantages and disadvantages of each method, which are summarized in Table 17.3 [2,102].

Inkjet printing

Since it was understood that liquid-like materials were more easily manipulated and allowed for the encapsulation of living cells, researchers leveraged technologies and commercialized devices that already had been optimized for the deposition of liquid materials. Inkjet printers, such as the HP Deskjet, were among the first devices to be utilized for bioprinting in tissue engineering since their modular design with interchangeable, refillable cartridges enabled multimaterial printing, which is a key feature of the most advanced modern bioprinters [106−109]. Also, translating the operation from printing documents to printing on substrates made *X*/*Y* operation and control straightforward, so control over the *Z*-axis could be added through high-tolerance, robotic operation of a piston or rod [108,110,111]. The inkjetting mechanism inherent to this technology provides a cell-friendly way of printing that is contactless and has little to no shear-stress force exerted upon the cell, avoiding these limitations that are associated with other bioprinting methods [31,64]. Some early attempts to utilize inkjet printing for tissue engineering applications involved vascular printing [46,106]. Specifically, printing vessel−like structures that support physiological flow conditions could be achieved by modifying the fine-deposition settings of the inkjet printer to allow printing over void space [46].

The latest innovations within inkjet bioprinting focus on overcoming material or process limitations, such as viscous materials clogging the nozzle and the inability to print high cell densities, and leveraging this method's strengths, which is its speed of deposition (∼10,000 drops/s) [112]. One study utilized a bioink composed of hMSCs suspended within a combination of gelatin methacrylate (GelMA) and PEG to increase the printability of GelMA, which has been shown to be useful in tissue engineering applications, but has high viscosity which limits its use in inkjet bioprinting [56,113]. Inkjet bioprinting has also successfully been applied to *in situ* bioprinting, which applies the printing process directly to a wound or defect and bypasses the need for a bioprinted construct to

TABLE 17.3 A brief summary of common 3D printing (3DP) and bioprinting methods.

	Inkjet	Extrusion	Laser-assisted bioprinting	Stereolithography
Advantages	Can print low-viscosity materials, fast fabrication speed, low cost, high resolution	Accessible and straightforward equipment operation, most capable of multimaterial printing, can print highest cell densities	High resolution, works with depositing biomaterials in a solid or liquid phase	Nozzle free—can avoid shear forces and potential clogging, printing time independent of complexity, high accuracy and resolution
Drawbacks	Unable to provide continuous flow due to jetting mechanism, poor construction of vertical structures, low cell densities	Only applicable for viscous bioinks, but high-viscosity/shear stress may have a cytotoxic effect	High cost of laser-based equipment, possible damage of cells due to laser irritation	UV/near-UV light source's cytotoxic effect, lack of easy multimaterial printing, damage to cells during photo-curing
Cost	Low	Moderate	High	Low
Resolution	~20–100 μm	~100–200 μm	~10–150 μm	<10 μm
Print speed	Fast	Slow	Medium	Fast
Supported viscosity	<10 mPa s	$30–6 \times 10^7$ mPa s	1–300 mPa s	No limitation
Cell density	Low	High	Medium	Medium
References	[2,102,103]	[2,102–104]	[2,102,105]	[2,12,102]

UV, Ultraviolet.

be separately cultivated and implanted [114]. By printing directly within a cartilage defect, researchers could control the exact position of chondrocytes with gene expression mimicking the native physiological environment and also avoid suturing or gluing implants, which typically yields inconsistent clinical results [82]. In an attempt to devise a more effective treatment for burns and skin wounds, amniotic fluid–derived stem cells were bioprinted within a fibrin-collagen gel directly into the wound site and yielded better wound closure and reepithelialization than bioprinting the gel alone [115]. These studies indicate inkjet-based bioprinting can be leveraged to treat clinical needs in novel ways that mimic physiological complexity.

Extrusion printing

Most bioprinting techniques under current investigation utilize direct deposition of cells and biomaterials through extrusion powered by pneumatic pressure, a piston, or a screw that provides deposition force. This is the most common technique employed by commercial bioprinting systems for tissue- and organ-printing studies [3,4,22]. Extrusion-based bioprinting (EBB) can be utilized in tandem with traditional polymer melt extrusion systems known as fused deposition modeling (FDM) that have been long employed for traditional supportive scaffold fabrication techniques in tissue engineering [40,43,51,116]. Due to the nature of FDM printing, including the relatively low cost of its components and the straightforward printing method with melted plastics, relatively large supportive scaffolds can be fabricated to create human-scale constructs of anatomical sizes with structural integrity [51].

Extrusion-based methods have been shown to bioprint constructs with the highest cell densities because of their ability to displace dense, concentrated bioinks from the print cartridge through the use of applied physical or pneumatic pressure [3,4,6,22]. Using this capability, some researchers engage in a "top-down" approach, simultaneously printing cells and complex architecture to create functional organ mimics—still, for achieving full-scale organ recapitulation, it has been estimated that 1–10 billion functioning cells, either primary in nature or fully differentiated into the desired physiological tissue phenotype, are needed to cross this therapeutic threshold, depending on the tissue type's characteristic cell density and size [117]. The need to sustain cellular proliferation in constructs of this size poses a new challenge, since diffusion of oxygen and nutrients is highly limited after several hundred micrometers below the tissue surface [117]. Instead of attempting to print everything at once, methods

to incorporate vasculature have been thoroughly investigated in the literature as a "bottom-up" approach to overcome the formation of a necrotic core inside thicker and/or denser cell constructs. These include direct printing and incorporation of open network channels and pores [51] and the use of sacrificial materials that create channels after their removal [118–121], as discussed in detail later. Bottom-up approaches rely less on constructing a rigid, finalized design of a vascular network, and instead rely on stimulation of endothelial cells to promote capillary and vasculature formation through incorporated growth factors or optimization of degradable matrix properties to allow for anastomosis to occur [122].

Laser-assisted bioprinting

Considering the large selection of material choices for cell-based printing, there is a variety not only in the materials themselves but also in the location where each resides on the spectrum of nonviscous, flowing materials to viscous, self-supporting gels that need minimal additional processing. Because of this diversity, it can be hard to apply certain materials within the realm of inkjet and EBB, as nozzle clogging has been seen in both printing modalities [26]. There is also an opportunity to improve resolution—for example, in EBB, resolution of the construct is generally limited to the diameter of the nozzle tip that is used for bioink displacement onto a substrate. LAB has been utilized to overcome these issues seen in other printers and increase resolution [123,124]. LAB works by utilizing the focused energy of a precisely positioned laser pulse to penetrate the optically transmitting side of the substrate that holds the cell-laden material, which then strikes an interlayer that prevents the full energy of the laser to reach the cell-laden material below but transfers enough energy to rapidly volatilize part of the lowest layer, causing a cell-containing droplet to be ejected downward onto the substrate below [105,123,124]. This multilayered material is known as a "ribbon," and the properties and thickness of the substrate, the interlayer, and the vaporizing polymer in which the cells reside combine to affect the energy transfer of the incident laser beam, and so all are important factors in modulating the volume, size, and speed of the resulting droplet [105,125].

Because of the microscopic control offered by galvanic mirrors and focusing lenses, LAB can be used to position individual cells on a substrate for finely constructed patterns [105,126]. Still, because LAB controls such a fine amount of volume with each laser pulse, larger constructs that are human scale would take an incredible amount of time to fabricate due to the number of layers that would be required in the *Z*-axis, and so this is more suited for the cell-patterning applications mentioned previously [125]. The latest innovations within LAB focus on utilizing its superior accuracy and precision in how cells and biomolecules can be deposited and patterned on a substrate. The relationship between the characteristics of the laser beam striking the transfer medium and the resulting shape and dynamics of the ejected bioink droplet pulse was elucidated and was shown to be capable of optimization for ensuring high viability in printed cellular constructs [64]. With this method, mesenchymal stromal cells have been printed within a collagen–hydroxyapatite mixture directly into the calvaria defects of living mice, and the results showed that different microscopic arrangements of cell constructs impacted bone tissue regeneration [127]. LAB's advantages were also leveraged in the study of cancer cell dynamics during angiogenesis through the creation of an ex vivo model via the precise printing of cancer cells over intact microvascular networks [128]. The relationships illustrated in these and similar studies were only possible through the exclusive degree of control over microscale cellular positioning that is offered by LAB.

Stereolithography

SLA has also been investigated to print living cells for translation into tissue engineering applications. SLA, which was the original 3DP method invented by Hull in 1984, is a method of constructing 3D objects through the curing of one layer of photopolymer at a time, which occurs when a UV layer traces a cross section of the object on the surface of a vat of prepolymer resin [101]. One of the advantages of SLA and dynamic light processing (DLP) printing, the adjacent 3DP technique that uses cross-sectional photomasks instead of a scanning laser, is their ability to cure hydrogels with a very high resolution, as discussed later [83,129]. Also, a recent development called "continuous liquid interface printing (CLIP)" combines the free radical-inhibiting effect of oxygen diffusing into the printing area with the speed of DLP has yielded unprecedented speeds for the construction of 3DP objects, though CLIP has yet to be shown with biological applications [129]. One disadvantage of light-initiated printing methods is that the process necessitates exposure to UV or near-UV light as well as how photoinitiators, monomers, or reactive diluents are in direct contact with cells. Depending on the specific chemistry or wavelength of curing light, these aspects involved in photo-curing resin have been shown to be potentially cytotoxic [29,34–36,94]. Limiting the duration of UV light exposure or using initiators that are activated by longer wavelengths of light can avoid damage to embedded cells [29,36]. Also, where EBB can easily print multimaterial constructs through the use of separate print cartridges, the structure of nearly all vat polymerization systems associated with SLA technology does not allow multiple

materials to be used at the same time. In spite of this, some studies have pushed through this barrier by changing resin vats between layers or printing with SLA around a previously extruded thermoplastic scaffold, creating patterned or layered constructs that possess different materials within a single structure [83–85].

As we discuss SLA for bioprinting, it is again important to note the distinction between a 3DP scaffold that is later seeded with cells and a bioprinted construct where the structural material also encapsulates living cells. The former has a long history of extensive investigation in the tissue engineering discipline, but the latter is more novel and has only become common in scientific studies of the past 5 years. In the earliest study attempting to combine modern SLA methods with encapsulated cells, researchers were able to demonstrate how this technique is able to fabricate constructs with well-distributed, living cells within the printed PEG-diacrylate (PEG-DA) material [86]. Later, another study investigated photo-curing hydrogels with a UV laser and demonstrated the potential for this SLA method by showing long-term viability of encapsulated cells [83]. More recently, a study utilized bioinks that were photo-crosslinked in the presence of visible light, avoiding the cytotoxic effects seen with shorter wavelengths of UV light [36,87]. This PEG-DA/GelMA-based system was used to fabricate constructs with 50 μm resolution with encapsulated, living cells that remained 85% viable after 5 days of culture [87]. With such high resolution and control, SLA also has the potential to make more clinically relevant constructs that mimic physiological complexity. One group presented a novel hybrid 3D printer (Hybprinter) that was capable of cross-linking a soft, cell-containing hydrogel material via DLP in the same layer that a supportive PCL framework was extruded, and a perfusable scaffold was fabricated for the investigation of better tissue engraftment and vascularization strategies [85].

Open source and commercial 3D printing systems

Though bioprinting machines built from the ground-up for a particular study may be able to create highly complex and innovative breakthroughs in complex scaffold fabrication [51], these results are hard to replicate without the same custom printer and consequently may represent a barrier to entry for further advancement by other research teams due to the high-cost and time-consuming effort involved in manufacturing bespoke robotic components and software. Open-sourced 3DP hardware aims to enable researchers and scientific advancement through knowledge-sharing communities and low-cost equipment. Two such printers that have a history of use in the literature are the RepRap and the Fab@Home, which have a modular design, can be easily modified to suit a particular printing study, and can even print iterations of themselves for continued low-cost maintenance [130,131]. There are accessories available that allow for polymer cross-linking, live dimensional error analysis, and direct printing into tissue defect models, making this system suitable for engineering complex tissues [37,38,131]. For example, the RepRap system has been used to tailor fiber diameter, pore size, and porosity in the printing of biodegradable scaffolds for bone tissue engineering [130]. Fab@Home-based printers have also seen used in tissue engineering applications [37,38,132], further demonstrating the research potential of low-cost, accessible 3DP technologies.

To continue this trend of cultivating a community committed to sharing resources and lowering barriers to entry for the sake of pursuing life-saving science, it would be beneficial for tissue engineering studies based on 3DP to be able to share the source code or 3D modeling files that were used to print that study's constructs. Without access to such files, including anatomical reconstructions or detailed organ microarchitectures that are converted to printable STL files, other research groups lacking the medical facilities or equipment to reference the same physiology encounter a barrier in recapitulating and validating their attempts to mimic the necessary tissue-specific, biologically relevant complexity [133]. To assist in overcoming this potential barrier for reproduction and scientific advancement, the National Institutes of Health founded the 3D Print Exchange in 2014 for open-source sharing of medically relevant, scientifically accurate files for anatomical models, custom labware, and replicas of biological molecules [133,134]. All of the materials on this database are uploaded by registered users that can be contacted for more information, and no payment is required for access or to obtain any files. The combination of this freely accessible resource and inexpensive printing systems, which can be modified by tissue engineering researchers and hobbyists alike, has the power to accelerate progress within the already revolutionary field of bioprinting.

While not as affordable as the open-source systems, commercial bioprinters have begun to appear on the market with widespread, international availability. Because of the cost-prohibitive nature of the laser-based equipment in LAB as well as both LAB and inkjet-based printing's limitations in fabricating larger-sized constructs with high cell densities, most of these products are extrusion-based machines [3,4,22]. Many of these printers come with features that enable researchers to pursue their studies with the accuracy and precision through capabilities such as self-calibrating printheads, proprietary software for the creation of 3D objects, and training programs as part of

the package. The ITOP system mentioned previously was one of the first in-depth demonstrations of printing multiple ink formulations of distinct composition in order to fabricate heterogenous, living tissue constructs [51]. Commercial printers are packaging these novel features and bringing them to widespread scientific audiences to enable researchers to achieve similar results by lowering the barriers to entry via singular, commercialized products. This accessibility is augmented by the significant decrease in price expected for many of these commercial products in the next 2 years [135]. One feature included by many bioprinting machines, which is necessitated by the environmentally sensitive nature of cells, is a sterilizable print chamber [136,137]. Some commercially available printers even include a full biosafety cabinet, which provides a level of biosafety engineering control required by certain biological studies [136,137]. Another useful feature for tissue engineers is the ability to use multiple materials to replicate heterogenous tissue complexity. Commercial printers have achieved this via the inclusion of multiple print heads that can be interchanged during printing with different functionality, including photocrosslinking, imaging, and extrusion of both soft hydrogel-like material and rigid support thermoplastic material [51,66,136,137]. The use of temperature control in a single print cartridge or a controllable temperature difference between the cartridge and print platform is another useful feature included in modern bioprinters [136,137]. It is especially useful in the case of investigating bioprinting with thermogelling materials, where low temperature is needed to maintain an extrudable state, alongside traditional thermoplastics, which require high temperature for printability [51,66,138]. The very latest, most advanced bioprinters push the envelope past traditional extrusion- or SLA-based methods into optical techniques that borrow from advanced, multiphoton microscopy. One example of this technology is the joint CELLINK−Prellis Holograph-X Bioprinter, which is capable of printing fully formed structures inside other already-polymerized layers through multiphoton polymerization with living cells embedded in the printable material [139]. This ability to prevascularize a bioprinted tissue and add complexity during fabrication is meant to break through barriers that have prevented researchers from making organ-like constructs that are orders of magnitude larger than previously investigated 3D spheroid constructs [2,139].

Print outputs: patterning, resolution, and porous architecture

By careful selection of tissue-specific design inputs and utilization of the appropriate 3DP systems, tissue engineers have created heterogeneous, multilayered scaffolds which more accurately recapitulate the biochemical and architectural properties of native tissue [11]. Nearly all tissues of interest to tissue engineers exist in a heterogeneous environment, in which blood vessels, connective tissue, and more interface with the target tissue to produce a milieu of biochemically and physically diverse properties [11]. Some tissues, such as the osteochondral unit, comprise multiple tissue phenotypes and zonal architectures, though traditional tissue engineering scaffolds have not fully mimicked the gradual transition in physical and biochemical properties, which occurs from bone to cartilage (see Fig. 17.3). Within these tissues, physical properties such as porosity and surface roughness may vary, in addition to the distribution of different biochemical cues such as growth factors and glycosaminoglycans. Thus the ability of 3DP and bioprinting to replicate these microenvironments relies on optimizing outputs such as the

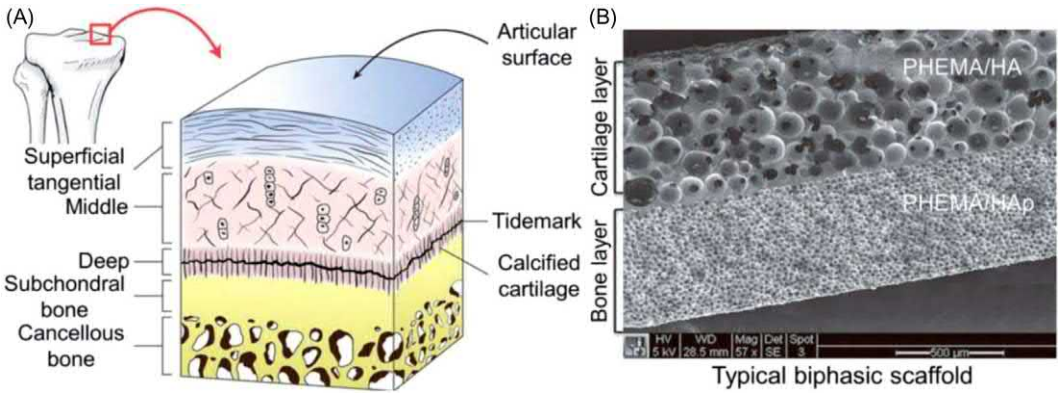

FIGURE 17.3 The osteochondral unit as an example of multilayered, heterogeneous tissue. (A) The distinct tissue phenotypes and physical architectures seen in the osteochondral unit, showing the gradual bone-to-cartilage transition. (B) A traditional bilayered scaffold mimicking the distinction between bone and cartilage, but not the transitional layers. *Adapted with permission from Li X, Ding J, Wang J, Zhuang X, Chen X. Biomimetic biphasic scaffolds for osteochondral defect repair. Regen Biomater 2015;2:221−8 [140].*

patterning of multiple inks, the deposition of high-resolution physical features, and the creation of interconnected porous architectures. After printing, it is crucial to then assess the physical features of the scaffold and confirm adherence to the intended architectures.

Printing/patterning of multiple inks

One key development in 3DP and bioprinting is the creation of printers that can deposit multiple inks in tandem. Commercial and custom-designed printers, as discussed previously, have now been developed with the capability to handle multiple material/ink cartridges or even multiple printheads for when different materials have distinct print conditions [11]. The ITOP system, for instance, uses a multicartridge module to deposit cell-encapsulated hydrogels, sacrificial cell-free hydrogels, and PCL in tandem and ultimately produce cell patterning in complex, vascularized constructs [11]. Ongoing research has focused on the development of printers that can switch more rapidly between ink cartridges and even mix ink formulations during printing, which can ultimately enable the generation of gradients in physical or biochemical properties within a single scaffold [141,142]. The usage of sacrificial inks, such as Pluronic F-127, can also provide structural support for architectures that would otherwise be susceptible to collapse during or after printing, and additionally can provide porous channels for nutrient and waste diffusion once the sacrificial material is removed [11]. Constructs thicker than 100−200 μm, which traditionally may have experienced cell death due to poor diffusion in the inner regions of the scaffold, can thus be printed with greater cell viability as a result of these porous channels [11]. These channels can furthermore be perfused with angiogenic factors or cells to produce vascularized tissue constructs with greatly enhanced viability [119]. The benefit of bioprinting and 3DP technologies is that the sacrificial materials can be deposited in highly defined distributions to produce well-organized vascular networks that resemble the distribution of vasculature in native tissue [119].

Another key component in developing complex, heterogeneous scaffolds is the patterning of growth factors and other biochemical cues by printing multiple inks within a single scaffold. For creating vascularized tissue constructs, it is important to have a stratified presentation of cues for angiogenesis and other tissue development [11]. For heterogeneous tissues, such as tendons, which contain transitions from bone to connective tissue to muscle, it is also important to generate spatial patterns of tissue-specific growth factors that mimic their organization in native tissue [11]. Growth factor patterning can be achieved by different means. One approach is to utilize multiple inks with different growth factor compositions and then to print the growth factors in their intended locations using the aforementioned multicartridge systems [11]. The growth factors themselves are usually loaded inside secondary vessels, such as poly(lactic-*co*-glycolic acid) (PLGA) microparticles, to allow for their sustained release kinetics after implantation of the scaffold [11]. Another approach is to print growth factor formulations at separate ends of a uniaxially oriented construct—for tissues such as nerves—and allow passive diffusion of the growth factors to generate growth factor gradients across the length of the construct [143]. In addition to spatial patterning, the temporal control of growth factor release is an ongoing area of research, as native tissue development involves different growth factor milieus at different timepoints of development [11]. While growth factor delivery from 3DP scaffolds has traditionally relied on the hydrolytic degradation of growth factor−containing microparticles, some research has now focused on methods to manually control growth factor release after scaffold implantation [11,144]. Plasmonic gold nanorods, for instance, maybe doped into PLGA microspheres so that spatiotemporally defined laser irradiation can be used after implantation to selectively heat nanorods and rupture biomolecule-containing microspheres [144]. Ultimately, it is of great interest for tissue engineers to continue developing new strategies for the spatiotemporal control of growth factor presentation so that these complex, 3DP scaffolds can more accurately recapitulate the dynamic biochemical environments seen in native tissue development.

Print resolution

Print resolution is a key parameter that determines not only the degree to which architectural components can be appropriately replicated but also to what degree growth factors or other biochemical cues can be patterned in the scaffold [104]. For the reconstruction of heterogeneous or vascularized tissues in particular, print resolution can determine the efficacy with which one can mimic the distinct physical and biochemical properties of different tissue types within a single construct [11]. The achievable resolution depends largely on the type of print technology and the associated limitations on droplet size, fiber diameter, etc., of the particular printer model. For extrusion-based printers the resolution is currently limited to about 200 μm and is effectively determined by the deposited fiber diameter, which in turn depends on needle/nozzle diameter [104]. In theory, smaller diameter needles will thus enable higher resolution printing, though the ability to achieve low enough viscosity to be printed through a small diameter needle depends on material/ink chemical composition and other properties such as molecular weight [145]. Thus the achievable resolution for an

extrusion-based system is determined by both the print system and the physical properties of the polymer ink itself. For inkjet systems, on the other hand, resolution is limited by the smallest physically achievable droplet size of approximately 1 pL, which results in an improved resolution compared to extrusion of about 20−100 μm [104]. LAB-printed droplets, on the other hand, can produce 80−100 μm resolution [105]. SLA and other light-based print methods, however, allow for the lowest achievable resolution of all the systems discussed so far, with SLA, in particular, being able to generate horizontal and vertical feature resolution as low as 1−2 μm [12]. Despite this the resolution of SLA-printed features can be compromised by shrinkage from residual cross-linking, and another notable drawback is that horizontal layers are necessarily uniform in material/ink composition due to spontaneous mixing of the preprint solutions—compromising any possibility of forming biochemical gradients in the same manner as extrusion- or inkjet-based techniques [12]. Overall, the granularity of growth factor and biomolecule patterning will depend on the resolution of print systems, which is an active an ongoing area of research for tissue engineers [104,146].

Porous architecture

The creation of interconnected porosity is an important output of 3DP, as tissue engineering scaffolds must allow for diffusion of waste and nutrients as well as cell migration during the regenerative process. Although 3DP can be used to create porous structures with high resolution, there are challenges associated with the maintenance of porous structures as printing progresses layer by layer. If the printed material is too soft or the fibers between subsequent vertical layers do not properly fuse, the resulting scaffold may experience poor structural integrity that leads to pore collapse [46]. These issues are typically exacerbated as the height and number of layers increase during the 3DP process, though they can sometimes be alleviated by coprinting rigid materials to support any soft materials susceptible to collapse. Cell-encapsulating hydrogels and natural polymers, such as gelatin, for instance, can be coprinted with rigid polyesters, such as PCL, so that the polyester provides maintenance of porous architecture [147]. Another potential issue is postprinting shrinkage or swelling of the material that changes pore size, which can particularly affect thermoresponsive polymers, such as gelatin [148]. Alternatively, sacrificial materials, such as Pluronic F-127 or carbohydrate glass, can be printed within hybrid scaffolds and subsequently removed to leave behind porous channels [118,119]. The benefit here is that channels with highly defined spatial organization can be created for the subsequent growth of vasculature or other tissue types within a heterogeneous construct [11]. Another method of introducing porosity is the usage of mesoporous materials that contain nanoscale pores within the material fibers themselves that are an order of magnitude smaller than the printed scaffold's macroporous network. These mesopores throughout the material fibers can be used as load bioactive molecules and enhance nutrient and waste diffusion in the printed construct [149]. Mesoporous bioactive glasses, in particular, have been 3D printed for the regeneration of bone tissue and present mesopores typically on the scale of 5−20 nm, compared to the main scaffold pores of several hundred microns in size [149,150].

Assessment of scaffold fidelity

During and after printing, it is important to assess the degree to which printed scaffolds resemble their digital models. Many 3D printers come equipped with camera systems that take top-down images of the scaffold in real time after each layer is printed [136]. These cameras are typically used in conjunction with laser-based distance sensors to automatically inform the printer of the location for the deposition of each layer and where to position the printhead. Essentially, the laser distance sensors scan the printed scaffold after each layer to attempt corrections for any misalignment of the printhead or discrepancies with the digital model. After the scaffold is printed the user can also assess any loss of fidelity from pore collapse or other issues by going through the images layer by layer and identifying any errors that propagate with layer deposition. The overall scaffold itself can be imaged and characterized by several means. First, SEM can be used to assess the surface morphology of printed scaffolds, as well as the fiber distribution and architecture at a very superficial level [12]. Before characterization, scaffolds are typically sputter coated with a thin layer of an electrically conductive material, such as gold, and then the scaffolds can be nondestructively imaged to produce high-quality images of scaffold surfaces. One of the primary limitations of SEM, however, is its limited depth of penetration. μCT instead can be used to generate full 3D images of high-density polymeric scaffolds—most often those incorporating metal or ceramic additives [149]. Another option is to incorporate fluorescent dyes within the inks, to allow for visualization of ink/material localization in the printed scaffold by fluorescent confocal microscopy [151]. Image analysis can then be used to quantify the spatial fidelity of material and dye deposition compared to the digital model. Regardless of the method used for scaffold visualization, it is essential for the user to verify the accuracy of both physical features as well as material localization for multimaterial scaffolds.

Printing applications: vascularized and complex, heterogeneous tissues

Using bioprinting techniques, tissue engineers have generated complex, heterogeneous scaffolds for various tissue types, ranging from osteochondral tissue [37] to skin [106], bladder tissue [152], liver tissue [153,154], heart valves [131], neural tissue [155], and more. For osteochondral tissue, some labs have printed gradients of osteogenic and chondrogenic biomolecules in order to mimic the spatial transition from bone to cartilage seen in native tissue. One study used a combinatorial approach of peptide gradients within a hydrogel that mimicked cell−cell or cell−matrix interactions seen in osteochondral tissue development and found that chondrogenesis varied spatially in these hydrogels based on the biochemical formulation [156]. To guide proper postprinting proliferation and differentiation, similar studies can serve as a blueprint for how to print biochemical factors in ways conducive to replicating native tissue complexity, which is one of the most difficult objectives of bioprinting [11]. Other strategies include the printing of gradients of osteogenic or chondrogenic growth factors in tandem with hydroxyapatite mineral gradients, in order to mimic the transition from bone-like to cartilage-like ECM [104,157]. For instance, the chondrogenic transforming growth factor beta 1 has been printed in an opposing gradient with hydroxyapatite, creating spatially gradated cues for chondrogenic and osteogenic development like that of the native osteochondral unit [157]. For neural tissue the bioprinting of neural stem cells and other progenitor cells has been combined with growth factor patterning to effect differentiation of delivered cells into glial cells, smooth muscle cells, and other relevant phenotypes [143,155,158]. For instance, axial gradients of neural growth factor and glial cell line−derived neurotrophic factor have been printed within silicon casts to provide directional cues for axons and Schwann cells, respectively, creating phenotypically appropriate tissue constructs for the repair of nerve gap injury [143]. To generate vascularized constructs, some groups have utilized sacrificial inks to generate vascular channels as discussed previously, while others have printed multiphasic scaffolds with vascular endothelial growth factor containing hydrogels dispersed throughout the construct [119,159].

Traditionally, printed scaffolds are cultured either in static conditions or in a perfusion bioreactor to produce some degree of cell attachment and/or differentiation prior to implantation. A few tissue engineers, however, have printed scaffolds in situ for intended application directly at the site of tissue defect, with notable examples, including skin [106] and meniscus [37]. The advantages of an in situ printing approach are that the scaffold can be implanted precisely at the site of defect with a minimized chance of infection, with adjustment to printing being made for the patient in real time, while the disadvantages are that scaffolds cannot be precultured for cells and that the conditions of printing are limited to those compatible with a physiological environment at material deposition.

Although significant progress has been made to address the biological functionality of 3DP scaffolds for specific tissues, several barriers remain for the implementation of 3DP strategies to human clinical applications. Regulatory approval is one critical prerequisite that can be challenging for tissue engineered products, especially those incorporating biological components such as cells [139]. The reader is encouraged to consult other chapters for a more in-depth discussion of the regulatory pathway. In addition, current technologies have yet to scale-up the output of these bioactive scaffolds in terms of sheer numbers. Further modification of the printing processes described in this chapter to allow for large-scale manufacturing will thus increase the potential for these technologies to address the high magnitude of clinical demand for implants.

Conclusion

3DP technologies, ranging from extrusion and inkjet printing to SLA and more, have enabled the creation of more highly biomimetic and complex scaffolds than those generated by traditional scaffold fabrication techniques. By selecting the appropriate design inputs, such as ink/material, cell type, and processing conditions, one can print scaffolds that recapitulate both the physical and biochemical cues of native tissue matrix. Thick, vascularized constructs and multiphenotype constructs, in particular, are now able to be fabricated more effectively due to advances in 3DP technology, and state-of-the-art custom printers have combined multiple processing techniques to print hydrogels, rigid synthetic polymers, and more, in tandem. As the field of bioprinting progresses, important considerations include the improvement of print resolution and handling of multiple inks, which maybe addressed by the development of even more powerful print systems across the techniques of inkjet printing, extrusion printing, SLA, and more. To improve biological activity of 3DP scaffolds, tissue engineers must also continue work on cytocompatible polymer-processing methods during printing, spatiotemporal control of biochemical cues, and the synthesis of novel bioactive and/or biocompatible scaffold materials. Furthermore, barriers remain for the implementation of these 3DP approaches in the clinic, including regulatory approval as well as the small scale in terms of scaffold numbers seen with current 3DP strategies. Tissue engineers will thus have to take these considerations in mind when developing new 3DP

systems and tissue applications. One important part of this process may be the translation of highly novel features seen in custom printers, such as multimaterial extrusion, to commercial printers in order to promote the utilization of these technological advances by more research groups within the field. Ultimately, 3DP and bioprinting, in particular, represent highly promising techniques for the fabrication of patient- and tissue-specific implants, and the development of new bioinks and innovative printer systems is ongoing.

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Abbreviations

μCT	micro-computed tomography
3DP	three-dimensional printing
CAD	computer-aided design
CLIP	continuous liquid interface printing
DLP	dynamic light processing
EBB	extrusion-based bioprinting
ECM	extracellular matrix
FDM	fused deposition modeling
GelMA	gelatin methacrylate
ITOP	integrated tissue−organ printer
LAB	laser-assisted bioprinting
MMP	matrix metalloproteinase
MSC	mesenchymal stem cell
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium
PCL	poly(ε-caprolactone)
PEG	poly(ethylene glycol)
PEG-DA	poly(ethylene glycol)-diacrylate
PLA	poly(lactic acid)
PLGA	poly(lactic-*co*-glycolic acid)
SEM	scanning electron microscopy
SLA	stereolithography
TCP	tricalcium phosphate
UV	ultraviolet

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Chapter 18

Biodegradable polymers

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Introduction

The design and development of tissue-engineered products have benefited from the clinical utilization of a wide range of biodegradable polymers. Newly developed biodegradable polymers and modifications of previously developed biodegradable polymers have enhanced the tools available for tissue-engineering applications. Insights gained from studies of cell−matrix interactions, cell−cell signaling, and organization of cellular components are placing increased demands on medical implants to interact with the patient's tissues in a more biologically suitable fashion. Whereas, in the 20th century, biocompatibility was largely equated with eliciting no harmful response, the biomaterials of the 21st century will have to elicit tissue responses that support healing or regeneration of the patient's own tissues.

This chapter surveys the universe of biodegradable polymers that may be useful in the development of medical implants and tissue-engineered products. Here, we distinguish between biologically derived and synthetic polymers. The materials are described in terms of their chemical composition, breakdown products, mechanism of breakdown, mechanical properties, and clinical limitations. Also discussed are the product design considerations for processing biomaterials into a final form (e.g., gel, membrane, and matrix) that will induce the desired tissue response.

In this chapter, we follow the official polymer nomenclature conventions adopted by the International Union of Pure and Applied Chemistry (IUPAC). According to these rules, the correct naming of a polymer requires the simple addition of the prefix "poly" in front of the monomer name, if the monomer name consists of a single word. For example, the polymer made of ethylene is simply polyethylene. There is no space between poly and ethylene. When the monomer name consists of two or more words, the entire monomer name must be enclosed in parentheses in order to avoid ambiguity. For example, the polymer made from trimethylene carbonate (TMC) is correctly named as poly(trimethylene carbonate) (PTMC). Note the absence of a space between the prefix poly and the parenthesis. Polymers made of amino acids are sometimes referred to as peptides, proteins, or poly(amino acid)s. When referring to a polymer that is composed of a single type of amino acid, IUPAC rules require this material to be named as a poly(amino acid). When referring to several such polymers, the plural of poly(amino acid) is poly(amino acid)s. Note the placement of the plural "s" outside of the parenthesis. When a polymer is made of several different amino acids, then that polymer would be referred to as a poly(amino acids). Note the placement of the plural "s" inside the parenthesis, indicating that it is a single polymer that contains more than one type of amino acid as monomer. Unfortunately, these rules are often violated in the contemporary literature.

Biodegradable polymer selection criteria

The selection of biomaterials plays a key role in the design and development of medical implants and tissue-engineering products. While the classical selection criterion for a safe, stable implant dictated choosing a passive, inert material, it is now understood that any artificial material placed into the body of a patient will elicit a cellular response [1,2]. In fact, some of the least chemically reactive materials, such as polyethylene or polysiloxane, can cause significant inflammatory responses. Therefore it is now widely accepted that a biomaterial must interact with tissue in a biologically suitable manner rather than act simply as an inert body. Consequently, a major focus of biomaterials research is harnessing control over cellular interactions with biomaterials. Researchers now tend to explore ways to manipulate the cellular response by including biologically active components in the design of

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biomaterials. Specific examples of such biologically active components include protein growth factors, antiinflammatory drugs, and gene delivery vectors [3].

While many biodegradable polymers have been developed for use as biomaterials, the selection of a polymer for a given application requires careful consideration of the specific cellular environment and interactions required. Such applications include

1. support for new tissue growth (wherein cell−cell communication and cells' access to nutrients, growth factors, and pharmaceutically active agents must be maximized);
2. prevention of cellular activity (where tissue growth, such as in surgically induced adhesions, is undesirable);
3. guided tissue response (enhancing a particular cellular response while inhibiting others);
4. enhancement of cell attachment and subsequent cellular activation [e.g., fibroblast attachment, proliferation, and extracellular matrix (ECM) production for dermis repair]; [4]
5. inhibition of cellular attachment and/or activation (e.g., platelet attachment to a vascular graft); and
6. prevention of a biological response (e.g., blocking antibodies against grafted cells used in organ replacement therapies).

Biodegradable polymers are applicable to those tissue-engineering products in which tissue repair or remodeling is the goal, but not where long-term material stability is required. Biodegradable polymers must also possess

1. manufacturing feasibility, including availability of sufficient commercial quantities of the bulk polymer;
2. the capability to be formed into the final product design;
3. mechanical properties that adequately address short-term function and do not interfere with long-term function;
4. low or negligible toxicity of degradation products, in terms of both local tissue response and systemic response; and
5. the capability to be formulated as a drug-delivery system in applications that call for prolonged release of pharmaceutically active compounds.

Biologically derived polymers

Biologically derived polymers are materials created by living organisms, as opposed to synthetic polymers, which are man-made. This distinction divides the universe of medically useful, biodegradable polymers into two large subgroups. However, the delineation between these groups is not always clear-cut. For example, glycolic acid is a natural metabolite and polyglycolide (PGA) is naturally produced by many organisms. However, glycolic acid can also be created synthetically from petroleum-derived starting materials. Currently, PGA is produced commercially by both fermentation and synthetic processes. Both production pathways result in the same final product, making PGA either a biologically derived or a synthetic polymer. A similar situation exists for polymers derived from hydroxybutyric and hydroxyvaleric acid. Commonly referred to as polyhydroxyalkanoates (PHAs), these polymers can be derived from either bacterial fermentation or purely synthetic processes. In this chapter, PHAs are included among the biologically derived polymers (since the most prevalent mode of commercial production is based on bacterial fermentation), while PGA and the closely related polylactide (PLA) are introduced to the reader as synthetic polymers (since the predominant mode of commercial production is currently based on petroleum-derived starting materials and synthetic polymerization reactions).

The biologically derived polymers can be further classified into peptides and proteins, polysaccharides, PHAs, and polynucleotides. Each of these subgroups will be discussed separately.

Peptides and proteins

Peptides and proteins are polymers derived from naturally occurring α-L-amino acids. Peptides are usually shorter chains (less than ∼100 amino acids), while proteins are longer chains (more than ∼100 amino acids). The amino acids are connected via hydrolytically stable amide bonds. Therefore these materials are usually degraded via enzymatic mechanisms. The major shortcoming of peptides and proteins as starting materials for the fabrication of any medical implant is their lack of processability. This term describes whether a polymer can be manufactured into a specific shape using any of the conventional polymer processing methods used in the plastics industry: compression molding, extrusion, injection molding, and fiber spinning. Another important limitation of peptides and proteins as biomaterials is their inherent immunogenicity. Any peptide or protein carries the risk of being recognized as foreign by the patient's immune system. For example, for many years, the safety of using bovine collagen was a hotly debated topic because of fears that the implantation of bovine collagen could provoke an immune response and predispose the patient to autoimmune diseases [5].

On the other hand, peptides and proteins can have outstanding biological properties and can facilitate designing biomaterials with desirable biological activity. This fact has been the driving force behind the long-standing interest in using peptides and proteins as starting materials for

TABLE 18.1 Proteins used as biodegradable biomaterials in medical implants.

Type of protein	Source	Function
Collagen	Isolated from cattle, fish, and other species	Key component of tissue architecture, which provides mechanical strength and supports cell attachment and growth. Used extensively as a tissue expander and bulking agent in cosmetic products
Gelatin	Partially hydrolyzed collagen	Used in the food industry, widely explored as a matrix for 3D cell culture and as a component of tissue-engineering scaffolds
Elastin	Isolated from cattle and birds	Key component of tissue architecture, which provides elasticity
Keratin	Isolated from skin, hair, and nails of sheep, cattle, and humans	Key structural component of outer skin, hair, and nails. Used as a matrix for cell growth and as a component in wound dressings and skin-care products
Silk	Isolated from insect larvae	Used in the textile industry because of its extraordinary strength. Also studied as a component of tissue-engineering scaffolds and as a cell culture substrate
Proteoglycans	Various tissue extracts	Used in research of cell–matrix interactions, matrix–matrix interactions, cell proliferation, and cell migration

medical implants or tissue-engineered products. Unfortunately, most peptides and proteins have mechanical properties that are not conducive for their use in medical implants, resulting in the usage of only a very small number of proteins as biomaterials (Table 18.1).

Collagen

Collagen is the major component of mammalian connective tissue, accounting for approximately 30% of all protein in the human body. It is found in every major tissue that requires strength and flexibility. Fourteen types of collagens have been identified; the most abundant being type I [6]. Because of its abundance and unique physical and biological properties, type I collagen has been used extensively in the formulation of biomedical materials [7]. Type I collagen is found in high concentrations in tendon, skin, bone, and fascia, which are consequently convenient and abundant sources for isolation of this natural polymer.

The structure, function, and synthesis of type I collagen have been thoroughly investigated [8]. Because of its phylogenetically well-conserved primary sequence and helical structure, collagen is only mildly immunoreactive [9]. However, many human recipients of medical or cosmetic products containing bovine collagen have antibovine collagen antibodies [10]. The clinical significance of this finding is not yet fully understood.

The individual collagen chains assemble into a triple α-helical structure known as procollagen, which can then be further modified to form larger organized structures. Collagen exists in tissue in the form of collagen fibers, fibrils, and macroscopic bundles [11]. For example, tendon and ligaments are comprised mainly of oriented type I collagen fibrils, which are extensively cross-linked in the extracellular space.

In vitro, collagen cross-linking can be enhanced after isolation through a number of well-described physical or chemical techniques [12]. Increasing the intermolecular cross-links

1. decreases biodegradation rate by reducing collagen's susceptibility to enzymatic degradation;
2. decreases the capacity of collagen to absorb water;
3. decreases collagen's solubility; and
4. increases the tensile strength of collagen fibers.

Collagen contains lysine residues with free amines, which can be used for cross-linking or modified to link or sequester active agents. These simple chemical modifications provide a variety of processing possibilities and consequently the potential for a wide range of tissue-engineering applications using type I collagen.

It has long been recognized that substrate attachment sites are necessary for growth, differentiation, replication, and metabolic activity of most cell types in culture. Collagen and its integrin-binding domains (e.g., Arg-Gly-Asp (RGD) sequences) assist in the maintenance of attachment-dependent cell types in culture [13]. For example, fibroblasts grown on collagen matrices appear to differentiate in ways that mimic in vivo cellular activity and to exhibit nearly identical morphology and metabolism [14]. Chondrocytes can also retain their phenotype and cellular activity when cultured on collagen [15]. Such results suggest that type I collagen can serve as tissue-regeneration scaffolds for any number of cellular constructs.

The recognition that collagen matrices could support new tissue growth was exploited to develop the original

formulations of artificial extracellular matrices for dermal replacements [1,2,16–18]. Yannas and Burke were the first to show that the rational design and construction of an artificial dermis could lead to the synthesis of a dermis-like structure whose physical properties "would resemble dermis more than they resembled scar" [18]. They created a collagen–chondroitin sulfate (CS) composite matrix with a well-described pore structure and cross-linking density that optimizes regrowth while minimizing scar formation [19]. The reported clinical evidence and its simplicity of concept make this device an important potential tool for the treatment of severely burned patients [20].

The advantageous properties of collagen for supporting tissue growth have been used in conjunction with the superior mechanical properties of synthetic biodegradable polymer systems to make hybrid tissue scaffolds for bone and cartilage [21,22]. These hybrid systems show good cell adhesion, interaction, and proliferation compared to the synthetic polymer system alone. Collagen has also been used to improve cell interactions with electrospun nanofibers of poly(hydroxy acids) such as PLA, PGA, poly(ε-caprolactone) (PCL), and their copolymers [21–25]. In addition, the combination of collagen scaffolds with biologically active entities, such as growth factors, platelet rich plasma [26], and various autologous or allogenic cell types [27], has provided significant opportunities for researchers to achieve tendon and ligament regeneration.

Gelatin

Gelatin is commonly used for pharmaceutical and medical applications because of its enzymatic biodegradability and biocompatibility in physiological environments [28–30]. Of the two types, acidic and alkaline gelatin, the former has an isoelectric point similar to collagen. The isoelectric point depends on its extraction procedure from collagen, and variations in it allow gelatin to bind with either positively or negatively charged therapeutic agents. Based on this fact, the acidic gelatin, with an isoelectric point of 5.0, could be used as a carrier for basic proteins in vivo, while basic gelatin, with an isoelectric point of 9.0, could be used for the sustained release of acidic proteins under physiological conditions. However, gelatin is liquid at temperatures above 30°C, so it must be cross-linked using compounds such as glutaraldehyde or genipin to generate hydrogels/scaffolds [31]. The advantage of gelatin as a carrier for controlled drug release is that the therapeutic agent can be loaded into the gelatin matrix under mild conditions. Gelatin hydrogels have been used as controlled release devices for a variety of growth factors known to enhance bone formation. For example, Yamada et al. successfully incorporated bFGF (basic fibroblast growth factor) into acidic gelatin hydrogels, which were implanted into a rabbit skull defect to allow for the localized release over 12 weeks [32,33]. In addition, gelatin scaffolds have been explored for cell culture and cell delivery [31].

Elastin

Elastin is an ECM protein and is most abundant in tissues where elasticity is of major importance, such as blood vessels (50% of dry weight), elastic ligaments (70% of dry weight), lungs (30% of dry weight) and skin (2%–4% of dry weight) [34,35]. It is important to note that elastin is not a single, well-defined molecule. Rather, elastin is used to describe a wide range of elastic peptide and protein sequences that exist in different lengths and with different compositions. A common feature of all elastin sequences is that they are rich in glycine, proline, and lysine. The basic structure–function–activity correlations of various elastin sequences were discovered by Urry et al. [36]. The elasticity and resilience of elastin stems from a combination of polymer chain recoil and a highly cross-linked structure [37].

In humans, elastin is synthesized early in life. By age 40 (approximately), elastin biosynthesis in humans slows down considerably. The appearance of skin wrinkles and other aging processes are directly related to the loss of elastin biosynthesis. This fact has been exploited as a marketing gimmick by cosmetic companies, who add elastin to a wide range of anti-aging products. However, there is overwhelming evidence that externally applied elastin is not able to pass through the skin and is not able to slow the loss of elastin from aging tissues [38].

Elastin is a highly versatile biomaterial that has inspired the imagination and curiosity of biomedical engineers. The ability of elastin to self-assemble into large supramolecular structures has been used to fabricate sponges, scaffolds, sheets, and tubes from human tropoelastin (elastin precursor) [39]. In addition, the use of solubilized elastin, tropoelastin, and elastin-derived peptides as surface coatings for synthetic polymers to improve the cellular response has been explored [37]. For example, poly(propylene fumarate) scaffolds coated with elastin had higher fibroblast and endothelial cell (EC) adhesion and proliferation than noncoated controls [40]. These elastin-based coatings adsorb onto a variety of synthetic polymers better than many other ECM proteins [41]. However, when elastin was used as a component in heart valve prosthetic devices, the deposition of calcium-rich precipitates was a significant problem. This process, often referred to as calcification, limited the utility of elastin-containing biomaterials in cardiovascular prosthetic implants [42]. This experience illustrates that the use of natural substances is not necessarily a guarantee for clinically successful device performance.

Keratin

Keratin is the name for a family of structural proteins that are abundant in the outer layer of human skin, in hair, and in nails. Keratin is rich in the amino acid cysteine and has the ability to self-assemble into bundles of fibers. Within these fiber bundles, individual strands are further cross-linked through disulfide (S−S) bonds involving the cysteine side chains. In this way, keratin forms particularly tough, insoluble structures that are among the strongest nonmineralized tissues found in nature. The only other nonmineralized tissue that resembles the toughness of keratin is chitin (the material found in the exoskeleton of insects and the outer shell of shellfish).

Since human hair is rich in keratin, this protein is added to many cosmetic (hair care) products. However, similar to the case of elastin-containing skin creams, keratin addition into hair-care products is primarily for marketing as externally applied keratin cannot penetrate into the hair shaft unless it has been hydrolyzed into short peptide sequences [43]. The exploration of keratin's properties also leads to the development of keratin-based biomaterials for use in biomedical applications. The unusual mechanical properties and strength of keratin and its ability to self-assemble were the driving force for these biomedical studies.

The history of keratin research illustrates another major challenge when using biologically derived polymers as biomaterials. There are a wide variety of keratins, which have been broadly categorized as hard and soft depending on their structure/function [44]. Thus depending on the animal/tissue source and extraction procedure used, the properties of the isolated keratin can vary dramatically [44]. The variability and irreproducibility of isolated protein samples is a general feature of most biologically derived polymers.

Early research focused on the preparation of protein films from keratin extracted from wool and human hair [45,46]. However, most laboratory-prepared keratin films were brittle and weak and did not share the outstanding strength of keratin formed in vivo [46]. Further research revealed that special preparation methods or the addition of glycerol as a plasticizer were required to form strong and flexible keratin films [45,47]. The feasibility of incorporating bioactive molecules, such as alkaline phosphatase into the keratin films for controlled release applications, has also been explored [46].

Keratin is also able to self-assemble into complex 3D architectures and induce cellular responses, which has been exploited to prepare scaffolds for tissue engineering. For example, keratin extracted from wool was used to form cell scaffolds for long-term cultivation [48]. Similarly, keratin extracted from human hair was used to generate hydrogels that facilitate nerve regeneration by enhancing Schwann cells attachment and proliferation and migration [49]. Finally, one of the most advanced biomaterial applications is the reported use of keratin extracted from human hair as a matrix for the regeneration of peripheral nerves [50].

Silk

On a weight basis, natural silk fibers can be stronger than high-grade steel. However, the strength of natural silk fibers is difficult to reproduce in the laboratory once pure silk has been extracted from the cocoons of silkworm larvae or from spiders' webs. Still, the unique mechanical properties of natural silk fibers have fascinated scientists for more than a century.

Consequently, researchers have explored its potential biomaterial applications and demonstrated that various silk protein preparations are biocompatible [51] and degradable [52]. In fact, the in vivo degradation rate of laboratory-made silk fibers can be tailored to be from months to years by modifying the processing procedure employed during the material's formation [52]. Silk can be thermally processed as it is stable below its degradation temperature (250°C or higher depending on processing method) [53]. In addition, silk biomaterials can withstand a variety of sterilization methods, such as ultraviolet (UV) and gamma irradiation, without altering their morphology or properties [54].

Min et al. investigated the potential of electrospun silk matrices for accelerating the early stages of wound healing [55]. In another study, chitin was blended with silk fibroin to fabricate composite fibrous scaffolds for skin tissue engineering. The rationale of these experiments was to combine the good mechanical properties of silk fibers with the wound healing effects of chitin [56]. The chitin/silk fibroin blends were electrospun to form nanofibrous matrices and evaluated for initial cell attachment and spreading [57]. In vitro, increased adhesion of keratinocytes was observed on chitin/silk blend matrices compared to pure chitin matrices, but the significance of these results for wound healing in vivo has not yet been established.

Although silkworm silk has been used to make medical sutures for decades [58], other commercially available silk-based medical products have only recently begun to emerge. For example, Allergan obtained a 510(k) clearance from the Food and Drug Administration (FDA) for a silk surgical scaffold (Seri) for soft tissue reinforcement of defect sites [59] and Sofregen obtained 510(k) clearance from the FDA for a solubilized silk injection to treat vocal fold paralysis in 2019 [60].

Proteoglycans

Proteoglycans (PGs) are a major component of the ECM. They consist of one or more glycosaminoglycan (GAG) chains that are attached, via a tetrasaccharide link, to serine residues within a core protein [61,62]. GAGs are long chains of repeating disaccharide units that are variably sulfated. There are four main classes of GAGs—hyaluronic acid (HA), CS/dermatan sulfate (DS), heparin/heparan sulfate (HS), and keratan sulfate (KS). PGs exhibit great structural diversity because each type of PG may contain different kinds, numbers, and lengths of GAG chains. In addition, each type of PG can have a different core protein structure or sulfate modifications in the disaccharide repeating patterns. Finally, PGs can be present in either monomeric or aggregate form. Both the core protein and the GAG chains of PGs play key roles in tissue remodeling, intracellular signaling, uptake of proteins, cell migration, and many other crucial functions in native tissues [61,62]. The fact that PGs seem to be involved in so many crucial cell signaling pathways is the main reason for the intense interest of biomedical engineers in understanding their properties.

To replicate the biological functions of PGs in tissue scaffolds, PGs or their GAG chains have frequently been grafted to the polymers used in fabricating tissue scaffolds or onto the surface of tissue-engineering scaffolds. Most notably, CS GAGs have been used to create collagen−GAG hybrid materials that seem to be particularly effective in skin regeneration [63].

PGs are sometimes used alone or in combination with other matrix proteins, such as fibrin, collagen, or chitosan, to create hybrid materials [64−66]. The rationale for exploring such complex mixtures is usually an attempt to "mix and match" biological properties with appropriate mechanical properties. For example, a mixture of PGs and ECM proteins is part of the widely used Matrigel scaffold [67]. Among the various combinations, collagen−GAG scaffolds are often preferred for tissue-engineering applications, because collagen provides a very cell-friendly matrix environment and the specific GAG used can affect (and regulate) cell behavior. Collagen−GAG hybrids can be readily sterilized using heat and can be manufactured with a variety of pore structures and a wide range of degradation rates [68]. In addition, a collagen−GAG hybrid is used in the first commercial tissue-engineered product, which is an artificial skin scaffold developed by Integra LifeSciences, a New Jersey company.

Biomimetic materials

Biomimetic materials are synthetic (man-made) materials that mimic natural materials or that follow a design motif derived from nature. In the previous section, a number of peptides and proteins were discussed. In general, peptides and proteins are isolated from natural sources and are therefore listed among the biologically derived polymers. However, significant research breakthroughs were made when scientists started to create mimics of natural polymers by semisynthetic or totally synthetic means. An excellent example of this research approach is the work of Urry et al., who used peptide synthesis methods to create artificial variants of elastin [69,70]. Using a combination of solid-phase peptide chemistry and genetically engineered bacteria, they synthesized several polymers that contained homologies of the elastin repeat sequences valine−proline−glycine−valine−glycine. These biomimetic polymers had better engineering properties than their natural equivalents, which made it possible to create films and fibers that could be further modified by cross-linking. The resultant films had intriguing mechanical responses, such as a reverse phase transition, which resulted in contraction with increasing temperature [70]. The exact transition temperature could be controlled by varying the polymer's amino acid composition [70]. Several medical applications are under consideration for this system, including musculoskeletal repair, ophthalmic devices, and mechanically/electrically stimulated drug delivery.

Other investigators, notably Tirrell and Cappello, have combined techniques from molecular and fermentation biology to create novel protein-based biomaterials [71−74]. These researchers had the innovative idea to create genetically engineered microorganisms that would produce the polymers that they wanted to study. In this way, completely new variations of biologically derived peptides and proteins could be prepared. These protein polymers were based on repeat oligomeric peptide units, which were controlled via the genetic information inserted into the producing bacteria. It has been shown that the mechanical properties and the biological activities of these protein polymers can be preprogrammed, which suggests a large number of potential biomedical applications [75].

Another approach to elicit an appropriate cellular response to a biomaterial is to graft active peptides to the surface of a biodegradable polymer. For example, peptides containing the RGD sequence have been grafted to various biodegradable polymers to provide active cell-binding surfaces [76]. Similarly, Panitch et al. incorporated oligopeptides containing the Arg-Glu-Asp-Val (REDV) sequence to stimulate EC binding for vascular grafts [77].

Polysaccharides

Polysaccharides are polymers made of various sugar (saccharide) units. The most common monosaccharides are

glucose and fructose. Sucrose is the chemical name of the widely used table sugar. Sucrose is a disaccharide composed of glucose and fructose. Scientists who are not experts in sugar chemistry are often unaware of the exquisite structural variability of these molecules. For example, the important human food, starch, and the structural polymer of all plants, cellulose, are both polymers of glucose. The only difference between starch and cellulose is the way in which the individual glucose units are linked together. When the many different saccharide isomers are combined using a variety of chemical bonds, the result is an extremely large number of structurally different polysaccharides. In fact, the chemistry of polysaccharides is as rich in diversity and variability as protein chemistry. It is therefore not surprising that various saccharides and polysaccharides play an important role in fine-tuning the responses of cells to their environment (Table 18.2).

As previously described, disaccharide and tetrasaccharide-linked polymers, such as GAGs and PGs, are critical in regulating key cell functions. In contrast, the industrially used polysaccharides (such as starch and cellulose) are polymers comprising exclusively various sugar (saccharide) units as monomers. These polysaccharides can be extremely large polymers containing millions of monomers and are mostly used in nature for cellular energy storage or as a structural material. As a general rule, most natural polysaccharides are not biodegradable when implanted in mammalian species due to a lack of digestive enzymes. Therefore without further chemical modification, most polysaccharides are not obvious material choices for use in biomedical applications.

Cellulose

Cellulose is the most abundant polymeric material in nature. In its most common form, it is a fibrous, tough, water-insoluble material that is mostly found in the cell walls of plants, mainly in stalks, stems, or trunks. Cellulose is the major component of wood. Cellulose is composed of D-glucose units that are linked together by β-(1→4) glycosidic bonds. In nature, cellulose is formed by a simple polymerization of glucose residues from a substrate such as Uridine diphosphate glucose [78]. Cellulose possesses high strength in the wet state [79]. The major commercial applications of cellulose are in the paper, wood, and textile industries [80] where millions of tons of cellulose are processed annually worldwide.

The major limitation of cellulose as a biomaterial is that it is not biodegradable, due to the lack of digestive enzymes for cellulose in humans [81,82]. However, a number of cellulose derivatives, such as methylcellulose, hydroxypropyl cellulose, and carboxymethyl cellulose, have been explored as biomaterials due to the useful material properties exhibited by these synthetically created derivatives [83]. The potential applications include drug-delivery implants, barriers for the prevention of surgical adhesions, or scaffolds in cartilage tissue engineering [84,85]. In vivo studies have been performed to assess the biocompatibility of a bacterial cellulose scaffold by subcutaneous implantation in rats [85]. While there were no macroscopic or microscopic signs of inflammation around the implants and no fibrotic capsule or giant cells were observed, the limited biodegradability of cellulose will most probably prevent the use of this material in most biomedical applications [85].

Starch

Whereas cellulose is composed of D-glucose units that are linked together by β-(1→4) glycosidic bonds, starch is composed of D-glucose units that are linked together by α-(1→4) glycosidic bonds. This change makes starch

TABLE 18.2 Widely investigated polysaccharides.

Type of polysaccharide	Source	Function
Cellulose	Cell walls of green plants	Main structural component of plants which keeps the stems, stalks, and trunks rigid
Starch (amylose and amylopectin)	Present in all staple foods	Important in plant energy storage
Alginate	Cell walls of bacteria	Protects bacteria from engulfment by predatory protozoa or white blood cells (phagocytes)
Glycosaminoglycans	Widely distributed	Cell–matrix interactions, matrix–matrix interactions, cell proliferation, and cell migration
Chitin/chitosan	Exoskeleton of insects, shells of crustaceans, cell walls of fungi	Structural component

digestible, thereby allowing its use as a major human nutrient. The chemistry of starch is complicated by the fact that starch consists of linear and branched chains, referred to as amylose and amylopectin, respectively [86]. The relative abundance of these two natural ingredients can significantly influence the material properties of starch. Consequently, hundreds of different starches exist, each with its own particular composition of amylose and amylopectin.

Starch can be totally water insoluble or partially soluble at room temperature, depending on the proportions of amylose and amylopectin present. Water-soluble starches can be dispersed in water and form clear solutions upon heating. Upon cooling, soluble starch forms a highly viscous solution at low concentrations and a stiff hydrogel at higher concentrations. This phenomenon is the basis of the thickening action of soluble corn starch, which is used extensively in the food industry.

Although enzymes present in the human gut can digest starch, it is not readily biodegradable when implanted into human tissues. The cellular energy storage polymer in humans is glycogen and not starch. Consequently, starch is not an obvious choice for biomedical applications. However, some starch-based polymers are biodegradable in human tissues and biocompatible, so several potential applications have been explored. Starch-based polymers were used to prepare scaffolds for cartilage regeneration, which did not perform better than other types of scaffolds [87]. Starch was also used for the design of implantable drug-delivery systems [88]. In spite of the research conducted by several laboratories worldwide, starch-based polymers are not likely to find major biomedical applications in the near future.

Alginate

Alginate is a natural anionic polysaccharide found in seaweed, which is composed of β-(1−4) linked D-mannuronic acid and α-L-guluronic acid units. Along its polymer chain, alginate has regions rich in sequential mannuronic acid units or guluronic acid units and regions, in which both monomers are equally prevalent.

The most important use of alginate in biomedicine is as a cell-compatible hydrogel. Alginate can form strong hydrogels in the presence of divalent cations (such as Ca^{2+} or Ba^{2+}) that interact with the carboxyl groups present along the alginate backbone to form ionic cross-links. In a typical procedure a solution of alginate is added to cells suspended in physiologic buffer solution. This mixture is then dropped slowly into a solution of calcium chloride. As each drop of the cell suspension touches the calcium chloride solution, the alginate forms a hydrogel that encapsulates and captures the suspended cells. This process has been used in vitro to encapsulate human articular chondrocytes in the presence of recombinant human bone morphogenetic protein 2 [89].

Alginate has a well-characterized structure, which allows for a range of comparative studies to be performed. Cells do not readily attach and grow on or within alginate hydrogels [90], which is a common feature of most unmodified polysaccharides. However, the carboxyl groups in its guluronic acid residues provide an easy handle for the chemical modification of alginate [91]. This feature makes it possible to attach biologically active ligands (such as the important RGD peptide) to the alginate backbone [91].

Alginate's major disadvantage is the difficulty of its isolation from contaminated seaweed, which leads to the presence of mitogenic, cytotoxic, and apoptosis-inducing impurities in the final processed material. Although such molecules can be removed by further purification steps, it is a time consuming and costly process [92].

Gellan gum

Gellan gum is another anionic polysaccharide, similar in utility profile to alginate. It can be easily processed into transparent gels that are resistant to heat. Gellan gum is not cytotoxic [93] and can be injected into tissues. It has been used in vivo in humans as an ocular drug-delivery vehicle [4]. Gellan gum is still relatively unknown in the biomedical community and only a few studies have explored this material for tissue engineering [94]. Like alginate, gellan gum can be used for the encapsulation and in vitro culture of cells [93]. Gellan gum hydrogels were able to support the development of nasal chondrocytes and injectable gellan gum hydrogels were efficient in the encapsulation and support of human articular chondrocytes while also enabling active synthesis of ECM components.

Glycosaminoglycans

GAGs, which consist of repeating disaccharide units in linear arrangement, usually include an uronic acid component (such as glucuronic acid) and a hexosamine component (such as *N*-acetyl-D-glucosamine). The predominant types of GAGs attached to naturally occurring core proteins of PGs include CS, DS, KS, and HS [95,96]. The GAGs are attached to the core protein by specific carbohydrate sequences containing three or four monosaccharides.

The largest GAG, HA (hyaluronan), is an anionic polysaccharide with repeating disaccharide units of *N*-acetylglucosamine and glucuronic acid, with many unbranched units ranging from several hundred to several thousand. HA can be isolated from natural sources (e.g., rooster combs) or via microbial fermentation [97].

Because of its water-binding capacity, dilute solutions of HA form viscous solutions.

Like collagen, HA can be easily chemically modified, for instance by esterification of the carboxyl moieties, which reduces its water solubility and increases its viscosity [97,98]. HA can be cross-linked to form molecular weight complexes in the range $(8-24)\times10^6$ g/mol or to form an infinite molecular network (gel). In one method, HA is cross-linked using aldehydes and small proteins to form bonds between the hydroxyl groups of the polysaccharide and the amino or imino groups of the protein, thus yielding high molecular weight complexes [99]. Other cross-linking techniques include the use of vinyl sulfone, which reacts to form an infinite network through sulfonyl-bis-ethyl cross-links [100]. The resultant infinite network gels can be formed into sheaths, membranes, tubes, sleeves, and particles of various shapes and sizes. Only minor cross-species variations have been found in the chemical and physical structure of HA [101]. In addition, it is biocompatible and elicits minimal inflammation, which makes it desirable as a biomaterial [102]. Its main drawbacks in this respect are its residence time and the limited range of its mechanical properties.

Because of its relative ease of isolation and modification and its superior ability in forming solid structures, HA has become the preferred GAG in medical device development. It has been used as a viscoelastic substance during eye surgeries since 1979 [103] and has undergone clinical testing as a means of relieving arthritic joints [104]. In addition, gels and films made from HA have shown clinical utility in preventing the formation of post-surgical adhesions [105–107]. The benzyl ester of HA, sold under the trade name HYAFF-11, has been studied for use in vascular grafts [108–113]. Finally, HA has recently seen widespread usage as a 3D printable bioink [114].

Chitosan

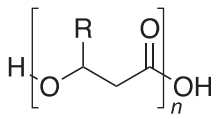
Chitosan is a biosynthetic polysaccharide that is the deacylated derivative of chitin. Chitin is a naturally occurring polysaccharide that can be extracted from crustacean exoskeletons or generated via fungal fermentation processes. Chitosan is a β-1,4-linked polymer of 2-amino-2-deoxy-D-glucose; thus it carries a positive charge from amine groups [115]. It is hypothesized that the major path for chitin and chitosan breakdown in vivo is through lysozyme, which acts slowly to depolymerize the polysaccharide [116]. The biodegradation rate of the polymer is determined by the amount of residual acetyl content, a parameter that can easily be varied. Chemical modification of chitosan produces materials with a variety of physical and mechanical properties [117–119]. For example, chitosan films and fibers can be formed using cross-linking chemistries adapted from techniques for altering other polysaccharides, such as treatment of amylose with epichlorohydrin [120]. Like HA, chitosan is not antigenic and is well-tolerated when implanted [121].

Chitosan has been formed into membranes and matrices suitable for several tissue-engineering applications [122–124] as well as conduits for guided nerve regeneration [125,126]. Chitosan matrix manipulation can be accomplished using the inherent electrostatic properties of the molecule. At low ionic strength, the chitosan chains are extended via the electrostatic interaction between amine groups, whereupon orientation occurs. As ionic strength increases and chain–chain spacing diminishes, the consequent increases in the junction zone and stiffness of the matrix results in increased average pore size. Chitosan gels, powders, films, and fibers have been formed and tested for such applications as encapsulation, membrane barriers, contact lens materials, cell culture, and inhibitors of blood coagulation [127].

Polyhydroxyalkanoates

PHAs are polyesters composed of β-hydroxy fatty acids such as of 3-hydroxybutyric acid and 3-hydroxyvaleric acid. These linear polymers provide intracellular energy storage by acting as a reserve of carbon and energy [128] in certain microorganisms. These polyesters are slowly biodegradable, biocompatible, thermoplastic materials [129,130]. Depending on growth conditions, bacterial strain and carbon source, the molecular weights of these polyesters can range from tens into the hundreds of thousands. Although the structures of PHA can contain a variety of *n*-alkyl side chain substituents (Structure 18.1), the most extensively studied PHA is the simplest: poly(3-hydroxybutyrate) (PHB).

Imperial Chemical Industries developed a biosynthetic process for the manufacture of PHB, based on the fermentation of sugars by the bacterium *Alcaligenes eutrophus* [131]. PHB homopolymer, like all other PHA homopolymers, is highly crystalline, extremely brittle, and relatively hydrophobic. Consequently, the PHA homopolymers have degradation times in vivo on the order of years [129,132]. Copolymers of PHB with 3-hydroxyvaleric acid are less crystalline, more flexible, tougher, and more readily processable [133]. However, they are still too hydrolytically stable to be useful in short-term applications where resorption of the degradable



STRUCTURE 18.1 General structure of poly(3-hydroxyalkanoate)s where R is an *n*-alkyl chain.

polymer within 1 year is desirable. PHB and its copolymers with up to 30% of 3-hydroxyvaleric acid are now commercially available under the trade name Biopol [133] and are mostly used as environment-friendly polymers that degrade slowly when disposed of in a landfill.

PHB has been found to have low toxicity, in part due to the fact that it degrades in vivo to D-3-hydroxybutyric acid, which is a normal constituent of human blood [134]. Applications of these previously tested polymers and others now under development include controlled drug release, artificial skin, and heart valves, along with industrial applications such as paramedical disposables [135–137]. Among the biomedical applications, sutures are the main product where PHAs are used, although a number of clinical trials for other applications may still be ongoing [138].

Polynucleotides

Gene delivery from the surfaces of tissue scaffolds represents a new approach to manipulating the local environment of cells [139]. Gene-therapy approaches can be employed to increase the expression of tissue inductive factors or block the expression of factors that would inhibit tissue formation [140]. A biomaterial can enhance gene transfer by localized expression of the genetic material and by protecting the genetic material against degradation by nucleases and proteases. Sustained delivery of DNA from a polymer matrix may transfect large numbers of cells at a localized site and lead to the production of a therapeutic protein that could enhance tissue development. For example, Yao investigated the potential of chitosan/collagen scaffolds with pEGFP-TGFβ1 as a gene vector candidate in cartilage tissue engineering [141].

Synthetic polymers

The concept of a polymer evolved from the study and commercial development of biologically derived macromolecules such as cellulose derivatives (celluloid, cellulose acetate) and vulcanized rubber in the 19th century. In 1907 the first totally synthetic polymer, Bakelite, was invented [142]. At that time the macromolecular structure of Bakelite and all other polymers was still not understood. It was only in 1922 that Hermann Staudinger proposed that the properties of polymers can be best explained by assuming that they consist of long chains of monomers linked together via regularly repeating bonds. The shortage of natural materials (in particular rubber) during World War II was the driving force behind the development of totally synthetic polymers. During World War II, nylon, Teflon, various polyesters, and synthetic rubber emerged. The tremendous improvements in virtually all consumer products since 1945 would not have been possible without the development of hundreds of specialized polymers.

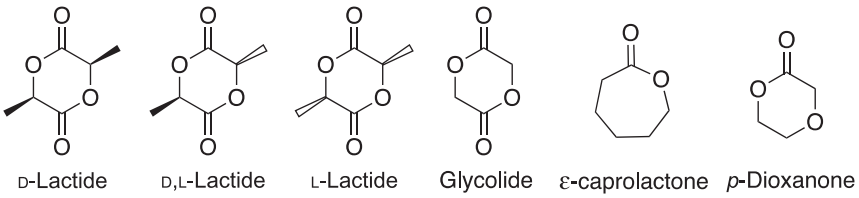
World War II left an additional legacy: the medical needs of millions of injured warfighters across the globe stimulated the development of new surgical procedures and innovative medical devices. Often, advances in the material sciences produced and enabled commensurate advances in medical practice. A new polymer, referred to as Vinyon N, was developed during World War II for use in parachutes. In 1952 vascular surgeons noticed the stretchiness and elasticity of this material and developed the first vascular graft using Vinyon N [143]. This effort became the starting point for the development of polymer-based medical implants and devices.

The systematic development of polymers for medical implants started with a focus on inert, biostable materials to be used in implants that lasted for the lifetime of the patient. It was only in 1969 that a biodegradable polymer, PGA, was used to create the first synthetic degradable suture line [144]—a breakthrough that ultimately ended the use of suture lines made from the intestines of animals. Due to the efforts of many research groups, a number of different polymeric structures and compositions have been explored as degradable biomaterials. However, commercial efforts to develop these new materials for specific medical applications have been limited. Thus detailed toxicological studies in vivo, investigations of degradation rate and mechanism, and careful evaluations of their physicomechanical properties have only been published for a very small fraction of those polymers. The following section is focused on a review of the most commonly investigated classes of biodegradable, synthetic polymers.

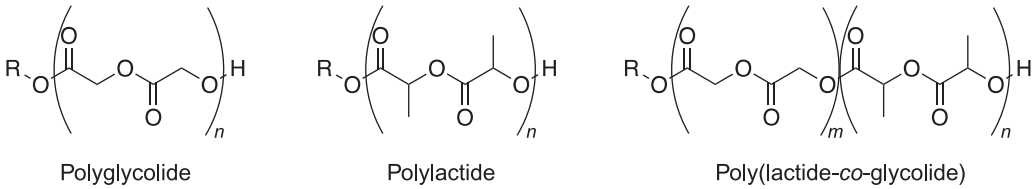
Aliphatic polyesters

Aliphatic polyesters made of hydroxy acids, such as glycolic acid, lactic acid, and ε-hydroxycaproic acid, have been used for a variety of medical product applications. As an example, bioresorbable surgical sutures made from poly(α-hydroxy acids) have been in clinical use since 1969 [144–146]. Other implantable devices made from these versatile polymers (e.g., internal fixation devices for orthopedic repair) are now part of standard surgical protocols [76,147,148].

Although polyesters can be synthesized by the polycondensation of hydroxy acids, such as lactic acid [149], it is difficult to achieve high molecular weights and control the molecular weight, molecular weight distribution, and architecture of the polymer using this process. In most cases, biodegradable polyesters are synthesized in a two-step procedure: first, the hydroxy acids are transformed into intramolecular lactones (Structure 18.2),



STRUCTURE 18.2 Cyclic esters used in ring-opening polymerizations.



STRUCTURE 18.3 Polyglycolide, polylactide, and their copolymer poly(lactide-*co*-glycolide).

which are then used as monomers in ring-opening polymerizations.

Aliphatic polyesters degrade via hydrolysis of the ester bonds, which results in a decrease in the polymer molecular weight of the implant [150]. This initial degradation occurs until the molecular weight of the resulting oligomers is less than 5000 g/mol, at which point the oligomers become water soluble and the degrading implant starts to lose mass as well. The final degradation and resorption of the polyester implants may also involve inflammatory cells, such as macrophages, lymphocytes, and neutrophils. Although this late-stage inflammatory response can have a deleterious effect on some healing events, these polymers have been successfully employed as matrices for cell transplantation and tissue regeneration [151,152]. The useful lifetime of implants made from these polymers is determined by the initial molecular weight, exposed surface area, crystallinity, and (in the case of copolymers) by the ratio of the monomers.

Aliphatic polyesters have a modest range of thermal and mechanical properties and a correspondingly modest range of processing conditions. The polymers can generally be formed into films, tubes, and matrices using such standard processing techniques as molding, extrusion, solvent casting, spin casting, and fused deposition modeling (3D melt printing). Ordered fibers, meshes, and open-cell foams have been formed to fulfill the surface area and cellular requirements of a variety of tissue-engineering constructs [76,147,153]. The aliphatic polyesters have also been combined with other components, for example, poly(ethylene glycol), to modify the cellular response elicited by the implant and its degradation products [154].

Polyglycolide, polylactide, and their copolymers

PGA, PLA, and their copolymer poly(lactide-*co*-glycolide) (PLGA) are the most widely used synthetic degradable polymers in medicine (Structure 18.3). Of this family of linear aliphatic polyesters, PGA has the simplest structure and is more hydrophilic than PLA. Since PGA is highly crystalline, it has a high melting point and low solubility in organic solvents. PGA was used in the development of the first totally synthetic absorbable suture [144]. The crystallinity of PGA in surgical sutures is typically in the range of 46%−52% [155]. Due to the hydrophilic nature and quick water uptake, surgical sutures made of PGA typically lose their mechanical strength over a period of 2−4 weeks postimplantation [156].

In order to adapt the materials properties of PGA to a wider range of possible applications, researchers undertook an intensive investigation of copolymers of PGA with PLA (Structure 18.3). Alternative sutures composed of PLGA are currently marketed under the trade name Vicryl [157]. Due to the presence of pendant methyl groups, PLA is more hydrophobic than PGA. High molecular weight PLA limits the water uptake of thin films to about 2% [155] and results in a rate of backbone hydrolysis lower than that of PGA [156]. In addition, PLA is more soluble in organic solvents than PGA.

It is noteworthy that there is no linear relationship between the ratio of glycolide to lactide and the physicomechanical properties of their copolymers. Whereas PGA is highly crystalline, crystallinity is rapidly lost in PLGA copolymers [158]. These morphological changes lead to an increase in the rates of hydration and hydrolysis. Thus copolymers tend to degrade more rapidly than either PGA or PLA [155,156].

Since lactic acid is a chiral molecule, it exists in two stereoisomeric forms that give rise to four morphologically distinct polymers. D-PLA (PDLA) and L-PLA (PLLA) are the two stereoregular polymers, D,L-PLA (PDLLA) is the racemic polymer obtained from a mixture of D- and L-lactic acid, and meso-PLA can be obtained from D,L-lactide. The polymers derived from the optically

active D and L monomers are semicrystalline materials, while the optically inactive PDLLA is always amorphous.

The differences in the crystallinity of PDLLA and PLLA have important practical ramifications. Since PDLLA is an amorphous polymer, it is usually considered for applications, such as drug delivery, where it is important to have a homogeneous dispersion of the active species within a monophasic matrix. On the other hand, the semicrystalline PLLA is preferred in applications where high mechanical strength and toughness are required, such as sutures and orthopedic devices [159–161].

PLLA and PDLA are semicrystalline polymers with a glass transition temperature (T_g) of approximately 60°C, and a peak melting temperature (T_m) of approximately 180°C [162,163]. PDLLA is amorphous with a T_g of 57°C [162]. Both semicrystalline PLLA and amorphous PDLLA polymers are rigid materials. Their Young's modulus and stress at break values are close to 3.5 GPa and 65 MPa, respectively. However, these polymers are relatively brittle with an elongation at break of less than 6% [164–166].

PLAs degrade by hydrolysis to form naturally occurring lactic acid. Degradation of the polymers starts with water uptake, followed by random cleavage of the ester bonds in the polymer chain. The degradation is throughout the bulk of the material [167]. Upon degradation the number of carboxylic end groups increases, which leads to a decrease in pH and an autocatalytic acceleration of the rate of degradation [168]. During the degradation of semicrystalline PLLA, crystallinity of the residual material increases as hydrolysis preferentially takes place in the amorphous domains [169]. In general, the rate of degradation and erosion of amorphous PDLLA is faster than that of PLLA [170].

Ikada et al. were the first to report on the stereocomplexation of enantiomeric PLLA and PDLA polymers during coprecipitation of mixed polymer solutions in nonsolvents [171]. Since then, they have shown that stereocomplexation of PLLA and PDLA can take place in dilute and concentrated solutions, during solvent evaporation and spinning, and during annealing of their mixtures prepared in the melt [172]. Other groups have also reported the formation of PLA stereocomplexes [173,174].

Upon stereocomplexation the melting temperature of the PLA stereocomplexes increases to about 230°C, which is 50°C higher than the enantiomeric PLLA and PDLA polymers. Stereocomplexation influences the mechanical properties of PLA films [167]. When stereocomplexes were formed from PLLA and PDLA, their films were stiffer, stronger, and tougher than films prepared from the enantiomeric polymers. In addition, under hydrolytic degradation conditions, the degradation of thin films of stereocomplexes of PLLA and PDLA is slower than that of the enantiomeric PLLA and PDLA homopolymers [175]. These findings are of great interest to biomedical engineers as they provide a simple way to increase the mechanical strength of these polymers with a concomitant increase in hydrolytic stability.

In addition, PLA, PGA, and their copolymers have been combined with bioactive ceramics, such as bioglass particles or hydroxyapatite, that stimulate bone regeneration while greatly improving the mechanical strength of the composite material [176]. It was also reported that composites of these polymers and bioglass are angiogenic (e.g., they supported the growth of blood vessels), thereby suggesting a novel approach for providing a vascular supply to implanted devices [177].

Some controversy surrounds the use of these materials for orthopedic applications. According to one review of the clinical outcomes for over 500 patients treated with resorbable pins made from either PGA or PLGA, 1.2% required reoperation due to device failure, 1.7% suffered from bacterial infection of the operative wound, and 7.9% developed a late noninfectious inflammatory response that warranted operative drainage [178]. This delayed inflammatory reaction represents the most serious complication of the use of PGA or PLA in orthopedic applications. The mean interval between device implantation and the clinical manifestation of this reaction is 12 weeks for PGA and can be as long as 3 years for the more slowly degrading PLA [178]. Whether avoiding reoperation to remove a metal implant outweighs an approximately 8% risk of severe inflammatory reaction is a difficult question; in any event an increasing number of trauma centers have suspended the use of these degradable fixation devices. It has been suggested that the release of acidic degradation products [glycolic acid for PGA, lactic acid for PLA, and glyoxylic acid for poly(*p*-dioxanone) (PDS)] contributes to the observed inflammatory reaction. Thus the late inflammatory response appears to be a direct consequence of the chemical composition of the polymer degradation products [178]. The incorporation of alkaline salts or antibodies to inflammatory mediators may diminish the risk of a late inflammatory response [179]. A more desirable solution to these problems for orthopedic (and perhaps other) applications requires the development of new polymers that do not release acidic degradation products upon hydrolysis.

Using biodegradable PGA mesh scaffolds and a biomimetic perfusion system, Niklason et al. successfully engineered small-diameter vessel grafts using either ECs and smooth muscle cells obtained from vessels in various species, or mesenchymal stem cells derived from adult human bone marrow [180,181]. In this approach, ex vivo culture resulted in the formation of a tissue-engineered vascular structure composed of cells and ECM, while the PGA degraded during the same period. Cellular material

was removed with detergents to render the grafts nonimmunogenic. Tested in a dog model, grafts demonstrated excellent patency and resisted dilatation, calcification, and intimal hyperplasia [182]. Finally, the semicrystalline character of PLLA means that it is well suited for the use in fused deposition modeling (3D melt printing) and it has been employed to generate a variety of complex architectures and 3D scaffolds for regenerative medicine [183].

Poly(ε-caprolactone)

PCL (Structure 18.4) is an aliphatic polyester that has been intensively investigated as a biomaterial. The discovery that PCL can be degraded by microorganisms led to the evaluation of PCL as a biodegradable packaging material; later, it was discovered that PCL can also be degraded hydrolytically under physiological conditions [184–186]. Under certain circumstances, cross-linked PCL can be degraded enzymatically, leading to what can be called enzymatic surface erosion [184,185]. Low molecular weight fragments of PCL are reportedly taken up by macrophages and degraded intracellularly, with a tissue reaction similar to that of the other poly(hydroxy acids) [186]. Compared with PGA or PLA, the degradation of PCL is significantly slower. PCL is therefore most suitable for the design of long-term, implantable systems, such as Capronor, a 1-year implantable contraceptive device [187].

PCL exhibits several unusual properties not found among the other aliphatic polyesters. Most noteworthy are its exceptionally low glass transition temperature of about −60°C and its low melting temperature of 57°C. Another unusual property is its high thermal stability. Whereas other tested aliphatic polyesters had decomposition temperatures (T_d) of 235°C–255°C, PCL has a T_d of 350°C, which is more typical of poly(ortho esters) than aliphatic polyesters [188]. Similar to PLLA, these thermal characteristics mean that PCL is well suited for fused deposition modeling (3D melt printing) and it has been used to prepare a variety of 3D scaffolds for tissue engineering [189,190].

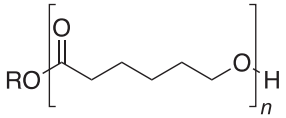
A useful property of PCL is its propensity to form compatible blends with a wide range of other polymers [191]. In addition, ε-caprolactone can be copolymerized with numerous other monomers (e.g., ethylene oxide, chloroprene, tetrahydrofuran, δ-valerolactone, 4-vinylanisole, styrene, methyl methacrylate, and vinyl acetate). Particularly noteworthy are copolymers of ε-caprolactone and the various lactide monomers, which have been studied extensively [185,192]. PCL and copolymers with PLA have been electrospun to create nanofibrous tissue-engineered scaffolds that show promise for vascular applications [23–25,193]. The toxicology of PCL has been extensively studied as part of the evaluation of Capronor. Based on a large number of tests, the monomer (ε-caprolactone) and the polymer (PCL) are currently regarded as nontoxic and tissue compatible materials. Early clinical studies [194] of the Capronor system were started around the year 2000 and resulted in a commercial implant used in Europe, but not in the United States.

Poly(p-dioxanone)

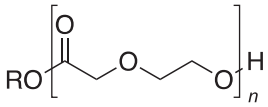
PDS is prepared by the ring-opening polymerization of *p*-dioxanone (Structure 18.5). It is a rapidly biodegrading polymer that was first commercialized by Ethicon in 1981 as a suture material [195]. When compared on a weight basis (e.g., per 1 g of implant), PDS releases degradation products that are less acidic than those released by PGA or PLA. The reduced acidity is a potential advantage for orthopedic applications, which led to the development of small bone pins (marketed as Orthosorb in the United States and Ethipin in Europe) for the fixation of fractures in nonload bearing bones [196,197]. However, PDS is softer, more flexible, and weaker than PLA/PGA, so it is better suited for soft tissue applications than for most orthopedic applications [195]. Thus it has been fabricated into rings for pediatric heart valve repair [198] and stents, such as the Ella stent, for use in the esophagus [199], trachea [200], and intestine [201]. In addition, PDS exhibits shape memory, so it is a promising material for tissue-engineering scaffolds for vascular grafts where it would provide kink resistance and rebound [195]. Finally, several academic laboratories have investigated PDS microparticles [202], micelles [block copolymer with poly(ethylene glycol)] [203], and electrospun nanofibers [204] for drug-delivery applications.

Poly(ortho esters)

Poly(ortho esters) are a family of synthetic degradable polymers that have been under development since 1970 [205,206]. Devices made of poly(ortho esters) can be formulated in such a way that the device undergoes surface erosion, which means that the polymeric device degrades at its surface only and thus tends to become thinner over



STRUCTURE 18.4 Poly(ε-caprolactone).



STRUCTURE 18.5 Poly(*p*-dioxanone).

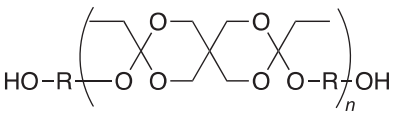
time rather than crumbling into pieces. Since surface-eroding, slab-like devices tend to release drugs embedded within the polymer at a constant rate, poly(ortho esters) appear to be particularly useful for controlled release drug delivery [207]; this interest is reflected by the many descriptions of these applications in the literature [208].

There are two major types of poly(ortho esters). Originally, poly(ortho esters) were prepared by the condensation of 2,2-diethoxytetrahydrofuran and a diol [209] and marketed under the trade names Chronomer and Alzamer. Upon hydrolysis, these polymers release acidic by-products that autocatalyze the degradation process, resulting in degradation rates that increase with time. Therefore Heller et al [210] synthesized a new type of poly(ortho ester) based on the reaction of 3,9-bis(ethylidene 2,4,8,10-tetraoxaspiro {5,5} undecane) with various diols (Structure 18.6), which do not release acidic by-products upon hydrolysis and thus do not exhibit autocatalytically increasing degradation rates. By selecting diols having different degrees of chain flexibility, polymers can be obtained that range from hard, brittle materials to materials that have a gel-like consistency. A drug release system for mepivacaine (a treatment for postoperative pain) was tested in Phase 2 clinical trials between 2000 and 2005 [206]. Finally, in 2016 the FDA-approved Sustol, which uses a fourth-generation poly(ortho ester)-based drug-delivery system (Biochronomer) to provide extended release (>5 days) of granisetron to prevent chemotherapy-induced nausea and vomiting [211,212].

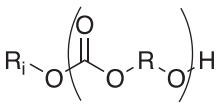
Aliphatic polycarbonates

Aliphatic polycarbonates (Structure 18.7) are biodegradable polymers that are promising biomaterials due to their nonacidic degradation products [213]. Similar to aliphatic polyesters, most aliphatic polycarbonates are prepared by the ring-opening polymerization of cyclic monomers [213,214]. By selecting appropriate monomers the hydrophobicity, degradability, viscoelasticity, and other properties of polycarbonates can be easily tailored [215,216]. Commercially available cyclic carbonate monomers include TMC, neopentylene carbonate, and 5-benzyloxytrimethylene carbonate, while numerous other carbonate monomers (>100) have been synthesized for specific applications [213,215]. The relative ease of preparing custom carbonate monomers makes them attractive candidates for introducing specific functionality into polyesters and polycarbonates [217]. For example, cyclic carbonate monomers with pendant functional groups, such as alkyls [218], alkynes [219], azides [220], coumarins [217], halides [221], protected hydroxyls [222], carbamates [223], carbohydrates [224], and dithiocarbonates [225], have been prepared and polymerized.

Aliphatic polycarbonates are typically amorphous with relatively low glass transition temperatures (T_g); the T_g for PTMC ranges from −17°C to −20°C depending on the molecular weight [226]. However, a few are semicrystalline, such as poly(neopentylene carbonate), which has a melting point of 107°C [227]. Notably, aliphatic polycarbonates, such as PTMC and poly(ethylene carbonate), have been observed to degrade by surface erosion in vivo [226,228] as opposed to the bulk degradation behavior observed for polyesters. The surface erosion in vivo is a result of macrophage-mediated enzymatic or oxidative degradation [229,230]. In addition, the in vivo degradation behavior of PTMC is dependent on the molecular weight with higher molecular weight polymers (>100 kg/mol) degrading faster than lower molecular weight polymers (<70 kg/mol) [226,231]. Consequently, high molecular weight PTMC has been explored for a variety of biomaterial applications including soft tissue regeneration [232–234], sutures [235,236], and drug delivery [237–239].



STRUCTURE 18.6 Poly(ortho ester) synthesized from DETOSU and a diol where R is any linear, branched, or cyclic alkyl chain. *DETOSU*, 3,9-bis(ethylidene 2,4,8,10-tetraoxaspiro {5,5} undecane).



STRUCTURE 18.7 Aliphatic polycarbonate initiated by an alcohol with alkyl chain, R_i, from a cyclic carbonate where R is an alkyl chain with optional pendant functionality.

Biodegradable polyurethanes

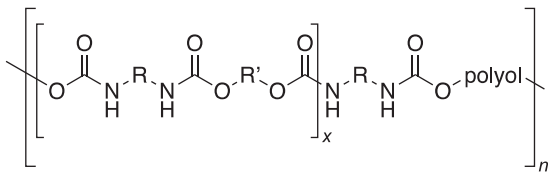
Polyurethanes contain a urethane moiety in their repeating unit and were first produced by Bayer in 1937. These polymers are typically produced through the reaction of a diisocyanate with a polyol. Conventional polyols are polyethers or polyesters. The resulting polymers are segmented block copolymers with the polyol providing a low glass transition temperature (i.e., <25°C) soft segment and the diisocyanate component, often combined with a hydrocarbon chain extender, providing the hard segment (Structure 18.8). A wide range of physical and mechanical properties have been realized with commercial polyurethanes.

Polyurethanes have been used in biomedical applications since the 1960s, particularly as a blood-contacting material in cardiovascular devices [240,241]. Intended as nonbiodegradable coatings, polyurethanes fell out of favor

with the failure of pacemaker leads and breast implant coatings. Subsequent studies, as reviewed by Santerre et al., have clarified much about the behavior of polyurethanes in biological systems [242]. Elucidation of the biodegradation mechanism and its dependence on the polyurethane structure and composition have led to the development of biodegradable polyurethanes for a variety of tissue-engineering applications such as meniscal reconstruction [243], myocardial repair [244], and vascular tissues [245]. The design of biodegradable polyurethanes has required alternative diisocyanate compounds since traditional aromatic diisocyanates are toxic and suspected carcinogens. Biodegradable polymers are made from biocompatible diisocyanates, such as lysine-diisocyanate or hexamethylene diisocyanate, that release nontoxic degradation products.

The urethane bond is essentially nondegradable under physiological conditions. Therefore biodegradable polyurethanes can only be obtained when the employed soft polyol segments are degradable [242]. As an example, aliphatic poly(ester urethane)s containing random 50/50 ε-caprolactone/L-lactide copolymer segments, 1,4-butanediol and 1,4-butanediisocyanate were synthesized and used to prepare porous structures for meniscus reconstruction [246]. In these polymers the biodegradation mechanism involves the hydrolytic cleavage of the ester bonds in the ε-caprolactone/L-lactide copolymer segments to form low molecular weight blocks of the nondegradable hard segments, which are ultimately excreted from the body via the liver and/or kidney.

An interesting application of polyurethanes was developed by Santerre et al. where fluoroquinolone antimicrobial drugs were incorporated into the polymer as hard-segment monomers [242]. This innovation led to the design of drug polymers (trade name: Epidel) that release the drug when degraded by enzymes generated by an inflammatory response. These polymers are an example of a "smart" system in that antibacterial agents are released only when inflammation is present. Once healing occurs, the enzyme level drops and the release of drug diminishes.



STRUCTURE 18.8 Segmented polyurethane prepared from a diisocyanate, OCN-R-NCO; a chain extender, HO-R′-OH; and a polyol building block.

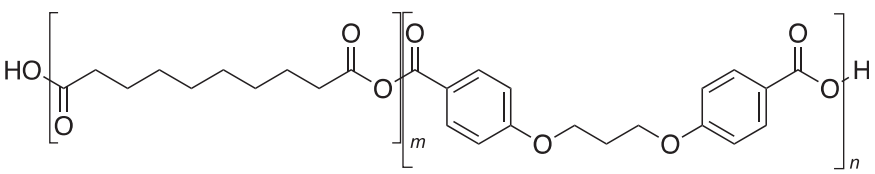
Polyanhydrides

Polyanhydrides (Structure 18.9) were first investigated in detail by Hill and Carothers [247] and were considered in the 1950s for possible applications as textile fibers [248]. Their low hydrolytic stability, the major limitation for their industrial applications, was later recognized as a potential advantage by Langer et al. [249], who suggested the use of polyanhydrides as degradable biomaterials. A study of the synthesis of high molecular weight polyanhydrides has been published by Domb et al. [250].

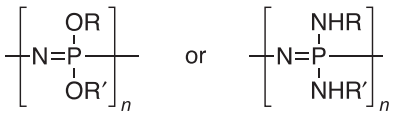
A comprehensive evaluation of the toxicity of the polyanhydrides showed that, in general, they possess excellent in vivo biocompatibility [251]. Their most immediate applications are in the field of drug delivery, although tissue-engineering applications are also being developed. Drug loaded devices are best prepared by compression molding or microencapsulation [252]. A wide variety of drugs and proteins, including insulin, bovine growth factors, angiogenesis inhibitors (e.g., heparin and cortisone), enzymes (e.g., alkaline phosphatase and β-galactosidase), and anesthetics have been incorporated into polyanhydride matrices, and their in vitro and in vivo release characteristics have been evaluated [253]. One of the most aggressively investigated uses of the polyanhydrides is for the delivery of chemotherapeutic agents. An example of this application is the delivery of BCNU (bis-chloroethylnitrosourea) to the brain for the treatment of glioblastoma multiforme, a universally fatal brain cancer [254]. For this application, polyanhydrides derived from 1,3-bis(4-carboxyphenoxy)propane and sebacic acid received FDA regulatory clearance in the fall of 1996 and are currently being marketed under the name Gliadel.

Polyphosphazenes

Polyphosphazenes (Structure 18.10) consist of an inorganic phosphorous-nitrogen backbone, in contrast to the commonly employed hydrocarbon-based polymers [255]. Consequently, the phosphazene backbone undergoes hydrolysis to phosphate and ammonium salts, with the concomitant release of the side group. Of the numerous



STRUCTURE 18.9 Example of a clinically relevant polyanhydride formed from two diacids, specifically sebacic acid and 1,3-bis(4-carboxyphenoxy)propane (repeating units *m* and *n*, respectively).



STRUCTURE 18.10 General structures for oxide and amine functionalized polyphosphazenes.

polyphosphazenes that have been synthesized, those that have potential for use in medical products are substituted with either amines of low p*K*a or activated alcohol moieties [256–258]. Singh et al. have modified the side groups to tune properties, such as the glass transition temperature, degradation rate, surface wettability, tensile strength, and elastic modulus, thereby enabling these polymers to be considered for a wider range of biomedical applications [259]. The most extensively studied polyphosphazenes are hydrophobic with fluoroalkoxy side groups [260]. In part, these materials are of interest because of their expected minimal tissue interaction, which is similar to Teflon.

Polymers of aryloxyphosphazenes and closely related derivatives have also been extensively studied. One such polymer can be cross-linked with dissolved cations, such as calcium, to form a hydrogel matrix because of its polyelectrolytic nature [261]. Using methods similar to alginate encapsulation, microspheres of this polymer have been used to encapsulate hybridoma cells without affecting their viability or their capacity to produce antibodies [262]. Interaction of these microspheres with poly(L-lysine) resulted in a semipermeable membrane that was capable of retaining the secreted antibodies [262].

Biodegradable polyphosphazenes have been explored for a range of biomedical applications, including nerve regeneration [263], tendon regeneration [264], and hard tissue-engineering scaffolds [265]. The delivery of drugs such as doxorubicin using polyphosphazenes has also been investigated [266]. Finally, an in vivo study of subcutaneously implanted samples of alanine-modified polyphosphazenes in a rat model demonstrated their biodegradability and resulted in a moderate inflammatory response after 2 weeks that had decreased to a mild inflammatory response after 12 weeks [267].

Poly(amino acids) and pseudo-poly(amino acids)

Since proteins are composed of amino acids, many researchers have tried to develop synthetic polymers derived from amino acids to serve as models for structural, biological, and immunological studies. In addition, many different types of poly(amino acids) have been investigated for use in biomedical applications [268]. Poly(amino acids) are usually prepared by the ring-opening polymerization of the corresponding *N*-carboxy anhydrides, which are in turn obtained by reaction of the amino acid with phosgene [269].

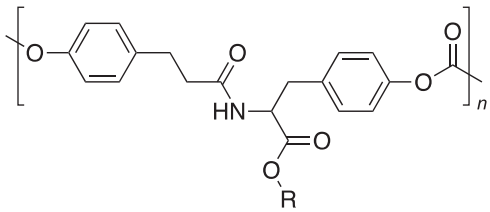
Poly(amino acids) have several potential advantages as biomaterials. The variety of amino acid structures allows a large number of polymers and copolymers to be prepared. In addition, amino acid side chains offer sites for the attachment of small peptides, drugs, cross-linking agents, or pendant groups to modify the physicomechanical properties of the polymer. Since these polymers release naturally occurring amino acids as the primary products of polymer backbone cleavage, their degradation products are expected to show a low level of systemic toxicity.

Poly(amino acids) have been investigated as suture materials [257], as artificial skin substitutes [270] and as drug-delivery systems [271]. Various drugs have been attached to the side chains of poly(amino acids), usually via a spacer unit that distances the drug from the backbone. Combinations of poly(amino acid)s and drugs that have been investigated include poly(L-lysine) with methotrexate and pepstatin [272] and poly(glutamic acid) with adriamycin and norethindrone [273]. Short amino acid sequences, such as RGD and Arg-Gly-Asp-Ser (RGDS), which are strong promotors of specific cell adhesion, have been linked to the polymer backbone to promote cell growth in tissue-engineering applications [274,275].

Despite their apparent potential as biomaterials, poly(amino acids) have actually found few practical applications. *N*-Carboxy anhydrides, the starting materials, are expensive to make and difficult to handle because of their high reactivity and moisture sensitivity. Also, most poly(amino acids) are highly insoluble and nonprocessable materials. Furthermore, the antigenicity of poly(amino acids) containing three or more amino acids limits their use in biomedical applications [268]. Finally, poly(amino acids) degrade via enzymatic hydrolysis of the amide bond, so it is difficult to reproduce and control their degradation in vivo, because the level of relevant enzymatic activity varies from person to person. Consequently, only a few poly(amino acids), usually derivatives of poly(glutamic acid) carrying various pendent chains at the γ-carboxylic acid group, have been identified as promising implant materials [276].

As an alternative approach, Kohn et al. replaced the peptide bonds in the backbone of synthetic poly(amino acids) by a variety of "nonamide" linkages such as ester, iminocarbonate, urethane, and carbonate bonds [277,278]. The term pseudo-poly(amino acid) is used to denote this new family of polymers, in which naturally occurring amino acids are linked together by nonamide bonds (Structure 18.11).

The use of such backbone-modified pseudo-poly(amino acids) as biomaterials was first suggested in 1984 [279]. The first pseudo-poly(amino acids) investigated



STRUCTURE 18.11 A poly(amide carbonate) derived from desaminotyrosyl tyrosine alkyl esters. This structure is an example of a new family of amino acid–derived polymers.

were a polyester from *N*-protected trans-4-hydroxy-L-proline and a poly(iminocarbonate) from tyrosine dipeptide [280,281]. Several studies indicate that the backbone modification of conventional poly(amino acids) generally improves their physicomechanical properties [277,282,283]. This approach is applicable to, among other materials, serine, hydroxyproline, threonine, tyrosine, cysteine, glutamic acid, and lysine; it is only limited by the requirement that the nonamide backbone linkages give rise to polymers with desirable material properties. Additional pseudo-poly(amino acids) can be obtained by considering dipeptides as monomeric starting materials. Hydroxyproline-derived polyesters [281,284], serine-derived polyesters [285], and tyrosine-derived polyiminocarbonates [286] and polycarbonates [287] represent specific embodiments of these synthetic concepts.

Combinations (hybrids) of synthetic and biologically derived polymers

Biologically derived polymers have important advantages over synthetic materials, which often include reduced toxicity and improved bioactivity. The reduced toxicity is typically due to the polymers deriving from molecules that naturally occur in the body [288]. The improved bioactivity refers to the ability of many biologically derived to elicit specific cellular responses. Collagen, for example, is a bioactive material because of its ability to support cell attachment, growth, and differentiation. However, the usefulness of biologically derived polymers is often limited by their poor engineering properties. Important disadvantages of biologically derived polymers are

1. high batch-to-batch variability due to complex isolation procedures from inconsistent sources;
2. poor solubility and processability, preventing the use of industrial manufacturing processes;
3. risk of contamination by pyrogens or pathogens;
4. poor or limited materials properties such as strength, ductility, elasticity, or shelf life; and
5. high cost.

In overall terms, synthetic materials offer a wider range of useful engineering properties (polymer composition, architecture, mechanical properties, etc.), but they generally fail to promote cell growth and differentiation to the same degree as some of the biologically derived polymers [289]. To address these shortcomings an obvious approach is to combine biologically derived polymers with synthetic materials. As a general rule, the purpose of such hybrid materials is to combine the bioactivity of biologically derived polymers with the superior engineering properties of synthetic materials.

As an example, a clinically useful cornea replacement material was obtained when collagen was incorporated into a synthetic, polyacrylate-based hydrogel [290]. Another example of this approach is provided by a hybrid material derived from silk fibroin (as the hydrophobic, bioactive material) and poly(vinyl alcohol) (PVA) (as the hydrophilic, synthetic component) [291]. PVA can be cross-linked under mild conditions by exposure to UV radiation. This cross-linking method allows PVA and fibroin to form a highly biocompatible hydrogel with adjustable engineering properties [291].

A final example of this approach is provided by a hybrid material composed of poly(*N*-isopropylacrylamide) (pNIPAM) as the synthetic component and various natural polysaccharides as the biologically derived component [292]. pNIPAM undergoes a thermally induced phase transition around 32°C that causes the soluble material to precipitate. The resulting product is liquid and injectable at room temperature and precipitates to form a hydrogel at body temperature, thereby facilitating the formation of a solid implant while eliminating the need for surgical insertion. Disadvantages of pNIPAM as a biomaterial include its inability to support cell growth and its lack of biodegradability. These disadvantages can be overcome by cross-linking pNIPAM with natural polysaccharide precursors, leading to cell-permissive formulations that will biodegrade in vivo.

Using polymers to create tissue-engineered products

The medical implant of the future will not be an artificial prosthetic device that replaces tissue lost due to trauma, disease, or aging, but a tissue scaffold that integrates with the surrounding healthy tissue and assists in the regeneration of lost or damaged tissue. As one possible implementation of this concept, the biomedical literature envisions porous sponge-like tissue scaffolds composed of bioactive polymers. These tissue scaffolds could either be preseeded with specific cell populations prior to implantation into the patient or be implanted first and then colonized by the patient's own cells, thereby leading to the regeneration of the desired tissue. In either case, one can formulate several design criteria to guide the biomedical engineer in

the process of selecting an appropriate biomaterial for the intended application.

First and foremost, one must be concerned with the bulk polymer properties. The biomaterial must have an appropriate range of physicomechanical properties, biodegradation rates, and biocompatibility appropriate for the intended application. Next, it must be possible to create the desired tissue scaffold shape and architecture using cost-effective fabrication methods. For example, electrospinning may be the preferred fabrication method to create an ECM-like scaffold that consists of nano-sized fibers. Since electrospinning requires that the biomaterial is soluble in nontoxic solvents, any insoluble biomaterial would not meet a key design requirement. Finally, it is important to consider the biological properties of the biomaterial. Tissue-engineering products require specific cellular responses. For example, in a bone regeneration scaffold, the ability to support the attachment and growth of osteoblasts in combination with the ability to adsorb and concentrate endogenous bone morphogenic protein on the polymer surface may be important design requirements. There are many additional requirements that impose further limitations on the selection of the biomaterial and/or the architecture of the implant. For example, whenever functional tissue has to form within the tissue-engineering scaffold, a lack of proper vascularization will impede a favorable tissue response. Details, such as pore size, pore structure, and pore connectivity, are often critical for promoting angiogenesis and hence vascularization of the scaffold [293].

Once an appropriately shaped tissue scaffold has been conceptualized, it is time to consider a wide range of secondary design criteria relating to the device's shelf life, ability to be sterilized and packaged for clinical use, and overall cost. Here, being able to create a terminally sterilized device is a factor often overlooked by academic researchers, who fail to consider the fact that most FDA-recognized sterilization methods cannot be applied to products that contain living cells. Thus the requirement to ensure device sterility has been a major hurdle in translating research concepts from the laboratory to clinical practice.

Barriers: membranes and tubes

Design formats requiring cell activity on one surface of a device while precluding transverse movement of surrounding cells onto that surface call for a barrier material. For example, peripheral nerve regeneration must allow for axonal growth, while at the same time precluding fibroblast activity that could produce neural-inhibiting connective tissue. Structures, such as collagen tubes, can be fabricated to yield a structure dense enough to inhibit connective tissue formation along the path of repair while allowing axonal growth through the lumen [294]. Subsequently, conduits composed of a silk fibroin scaffold coupled with biologics have shown improved performance [295]. Similarly, collagen membranes for periodontal repair provide an environment for periodontal ligament regrowth and attachment while preventing epithelial ingrowth into the healing site [296]. Antiadhesion formulations using HA, which prevent ingrowth of connective tissue at a surgically repaired site, also work in this way [105].

Gels

Gels are used to generate a specialized environment for cells by either providing a hydrogel scaffold on which they can grow or encapsulating them in a protective isolated environment. For example, collagen gels for tissue engineering were first used to maintain fibroblasts, which were the basis of a living skin equivalent [297]. Gels have also been used for the maintenance and immunoprotection of xenograft and homograft cells, such as hepatocytes, chondrocytes, and islets of Langerhans, used for transplantation [298–300]. Semipermeable gels have been created to limit cell–cell communication and interaction with surrounding tissue, and to minimize the movement of peptide factors and nutrients through the implant. Injectable biodegradable gel materials that form through cross-linking in situ show promise for the regeneration of bone and cartilage [301]. The use of biodegradable polyester dendrimers (highly branched, synthetic polymers with layered architectures) to form hydrogels for tissue-engineering applications such as corneal wound sealants has also been discussed [302]. In general, nondegradable materials are used for cell encapsulation to maximize the long-term stability of the implant. In the future, however, it may be possible to formulate novel "smart" gels, in which biodegradation is triggered by a specific cellular response instead of simple hydrolysis.

Matrices

It has been recognized since the mid-1970s that 3D structures are an important component of engineered tissue development [16,17]. Yannas et al. were the first to show that pore size, pore orientation, and fiber structure are important characteristics in the design of cell scaffolds. Several techniques have subsequently been developed to form well-defined matrices from synthetic and biologically derived polymers, and the physical characteristics of these matrices are routinely varied to maximize cellular and tissue responses [303–306]. Examples of engineered matrices that have led to several resorbable templates are oriented pore structures designed for regeneration of trabecular bone [307,308].

Conclusion

Research using currently available biomaterials and research aimed at developing novel biodegradable polymers have both helped to advance the field of tissue engineering. Throughout most of the 20th century, research and development efforts focused on a small number of biodegradable polymers with a history of regulatory approval, thereby making PLA the most widely used biodegradable polymer. Research in the 21st century aims to develop advanced biodegradable polymers that elicit predictable and useful cellular responses. To achieve this goal, current research efforts focus on creating bioactive materials that combine the superior engineering properties of synthetic polymers with the superior biological properties of natural materials. The extremely complex material requirements of tissue scaffolds pose significant challenges and require a continued research effort toward the development of new bioresorbable polymers.

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Chapter 19

Three-dimensional scaffolds

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Introduction

A key concept in tissue engineering (TE) is using material-based porous three-dimensional (3D) scaffolds to provide physical and structural support and deliver micro-environmental cues to cells to enable or facilitate tissue formation [1]. Through decades of investigations, 3D scaffolds have evolved to serve complex functions with dynamic and programmable features at nano-, micro- to macro-scales in various TE applications. Scaffolds can be seeded with different types of cells, including stem cells, progenitors, mature differentiated cells, or cocultures of cells for de novo tissue construction in vitro; scaffolds can also be directly implanted in vivo to deliver soluble/insoluble and temporal/spatial cues to guide the regeneration of defected tissue in situ. While their functions vary with specific TE approaches and clinical needs, scaffolds may potentially coordinate biological events on time and length scales ranging from seconds to weeks and nanometers to centimeters, respectively [2]. One important theme in the research of 3D scaffolds is to advance the material synthesis and fabrication techniques to enable the construction or regeneration of functional tissue. On the other hand, efforts should be directed to understanding the correlations between scaffold properties and its biological functions in directing cell and tissue responses, which constitute the important foundation for deriving design principles for 3D scaffolds.

During the biological processes of tissue development, cells constantly decode and release different morphogenetic factors into their surroundings. In response, cells make decisions on how to divide, differentiate, migrate, degrade/produce extracellular matrix (ECM), and orient themselves. Based on our understanding of reciprocal cell−ECM interactions in native tissues, it should theoretically be possible to design scaffolds to mimic the regulatory function of the natural ECM and promote tissue formation via programing one or more chemical/physical properties in scaffolds. Living cells and tissues, however, are not simple, linear systems. The complex roles that ECM structures play in orchestrating cellular and tissue processes are exhibited with spatial and temporal dynamics and precision. Complexity may also come from the context of an in vivo physiological condition, which generates variables not only from mechanical or electrical processes but also local/systemic pathological responses. Indeed, the dynamic superposition of environmental factors with the function of scaffolds can profoundly impact the outcome of the tissue-formation process. Nevertheless, correlations between individual scaffold properties and certain aspects of the tissue-formation process (e.g., cellular phenotype/organization, ECM production, emergence of tissue structure, and recovery of tissue function) can be derived and applied to the optimization of scaffold designs [3−6].

In this chapter, based on our current understanding of the correlation between scaffold properties and functions in promoting tissue formation, we summarize basic scaffold design approaches and essential principles that may guide the rational multifunctional design of scaffolds for TE (Fig. 19.1A). Design variables are decoupled and analyzed in terms of how they have been controlled using different materials and fabrication techniques, and what implications they have in regulating the tissue-formation process. From an engineering standpoint, it is desirable that scaffold properties and functions can be reproducibly integrated within a scaffold construct through standardized and customizable fabrication process, such that different types of cellular and tissue events can be coordinated/controlled and patient-specific variations and needs can be addressed.

Three-dimensional scaffold design and engineering

An understanding of the fundamental scaffold properties and functions involves hierarchal design of temporal and/or

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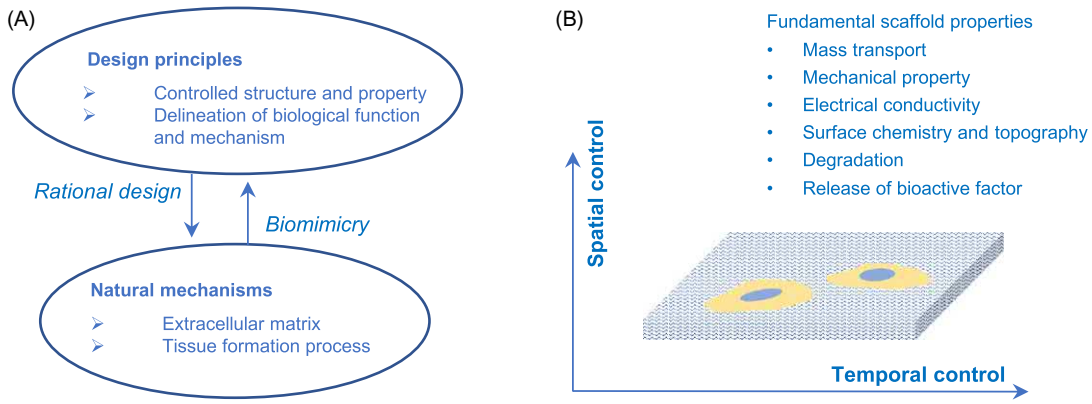

FIGURE 19.1 **A)** Design of TE scaffolds involves the rational approach through elucidation of structure-property-function relationships in scaffolds and the biomimicry approach through emulating natural mechanisms of ECM during tissue formation process. **B)** A TE scaffold holds fundamental properties that are essential to its function—spatial as well as temporal control of these properties in 3D environment enables building scaffolds with dynamic and instructive functions.

spatial parameters in scaffolds at multiple length scales and investigation of cellular and tissue responses under different conditions (Fig. 19.1B). At any given time point the scaffold properties will provide an initial set of environmental conditions for cells and tissues at the onset of in vitro seeding (i.e., time zero) or during the acute phase of the host response following direct implantation in vivo. These fundamental material properties are basic scaffold variables for mediating cell behavior and tissue responses and they typically include mass transport (pore architecture), mechanics, electrical conductivity, and surface chemistry and topology (Fig. 19.1B). TE involves dynamic processes in 3D space. On the temporal and spatial axes, the scaffold properties may be varied, patterned, or programed. Temporally, the initial scaffold properties and associated functions would evolve over time in response to the seeded or infiltrating cells in an in vitro/in vivo environment. The degradation of scaffold and release of bioactive factors are two active scaffold properties that can modulate the construct and local microenvironment over time and will be discussed under the "Temporal control" section. Spatially, all properties of fundamental materials can be generated with anisotropic patterns and constraints to guide cell orientation, cell−cell interaction, and tissue morphogenesis. Designing and enabling spatial control therefore presents a major challenge in TE given the complicated hierarchal organization of cells, ECM and functional units in all tissue types. As all the fundamental scaffold properties and functions act in an interdependent way, the investigation and fabrication of 3D scaffolds entail decoupling of variables and understanding how each aspects of a scaffold correlate with the biological function. These scaffold properties can then be integrated to yield optimized constructs for different TE applications.

Mass transport and pore architectures

As a porous 3D substrate, the scaffold provides conditions that determine how fluids, solutes, and cells move in and out of the tissue construct. The mass transport processes are correlated with the scaffold chemical compositions and the geometrical parameters of its pores or voids—characterized mainly by pore size, porosity, pore interconnectivity/tortuosity, and surface area. The pore architecture of a scaffold may also change due to chemical disruption [e.g., pH dependence of the hydrolysis of poly (lactic-*co*-glycolic acid) (PLGA) scaffolds] and modulation by biological components. These factors interdependently affect the diffusive or convective behavior of soluble nutrients, growth factors, and cytokines. Compared to the vascularized tissues containing convective flows, diffusion is often the primary mass transport mechanism in engineered tissue constructs cultivated in vitro and prior to inosculation with the host vasculature following implantation in vivo. In the environment with fluidic dynamics, permeability has been used as an effective parameter to characterize the property of a scaffold to transport water, small molecules, and proteins [7,8]. Permeability of a scaffold can be measured through experiments combined with computational methods to derive the permeability coefficients that reflect the hindered movement of solutes and permeation of fluids through porous scaffolds [9].

The structural characteristics of the pores and void spaces in scaffolds are also the primary variables that govern the initial cell distribution and organization in scaffold. Once a scaffold is impregnated with cells, the solute diffusion and distribution profiles are influenced by the cellular environment as well as the pore architecture of the scaffold. In metabolically active tissues in vivo,

most cells reside within 100 μm of a capillary. In engineered tissue constructs cultivated in static culture without medium perfusion, neo-tissue formation is generally limited to the peripheral 100−200 μm of the scaffold due to diffusion limitations [10−12]. Subsequently, the organization and density of cells in this peripheral region can further influence the distribution and availability of nutrients to cells within the scaffold interior [13]. Although certain types of cells may tolerate nutrient deficiency to some extent (such as chondrocytes under hypoxia), the induction of rapid vascularization following implantation is considered essential for establishing nutrient exchange and retaining a viable cell population within the construct.

One main parameter that affects the efficiency of initial cell impregnation is the pore size of the scaffold. The resulting geometries and spatial characteristics of individual cells or cell aggregates within the void space of scaffolds direct the subsequent cell proliferation, differentiation, and tissue formation events. As observed in the nonwoven fibrous scaffolds made of poly(ethylene terephthalate) and seeded with human placenta trophoblast cells in vitro, the size of cell aggregates increased with larger pore volumes between fibers, but larger cell aggregates exhibited attenuated differentiation and suppressed proliferation activities compared to smaller ones [14,15]. In another study, in the genipin-cross-linked gelatin scaffolds, chondrocytes demonstrated dedifferentiation in pores smaller than 200 μm but better proliferation and ECM production in the scaffolds with pore size between 250 and 500 μm [16].

Cell transport and vascularization as a result of scaffold pore size can also affect the tissue types and tissue-formation process in scaffolds. When bone morphogenetic proteins were loaded into honeycomb-shaped hydroxyapatite scaffolds to induce osteogenesis, it was found that smaller diameters (90−120 μm) induced cartilage followed by bone formation, whereas those with larger diameters (350 μm) induced bone formation directly [17]. The difference was likely caused by the different onset time of vascularization and cell differentiation.

Optimal scaffold pore sizes have been suggested for regenerating various types of tissues in vivo. For example, the critical pore size was found to be above 500 μm for rapid fibro-vascularization in poly(L-lactic acid) (PLLA) scaffolds with cylindrical pores [18]. In the ECM analog scaffolds made of cross-linked collagen and glycosaminoglycans (GAGs), the average pore diameters required to induce dermis and peripheral nerve regeneration were within 20−120 and 5−10 μm, respectively [19]. In bone engineering, early studies by Hulbert et al. showed that a minimum pore size of 100 μm was required to allow bone tissue ingrowth in ceramic scaffolds [20]. Further investigations found that larger pore sizes (e.g., > 100 μm) may favor higher alkaline phosphatase activity and more bone formation [21,22]. In recent years, mesenchymal stromal cells (MSCs) from animal and human sources have been intensively investigated for tissue regeneration applications through different scaffolds. The pore size may also range from tens to hundreds of micrometers depending on the specific cell type and scaffolds. A summarization that may suggest optimal pore size and porosity in different TE scenarios can be found in the review by Loh and Choong [23].

In addition to pore size, cell transport behavior such as diffusion, attachment, and migration are also affected by a number of other basic geometrical parameters of scaffolds, including porosity (the fraction of pore volume), pore shape, pore interconnectivity, and surface area [24]. It is therefore pivotal to elucidate how the architecture of pores relates to the biological response of cells and tissues. Rapid prototyping or solid free-form fabrication allows the generation of highly controlled scaffold structures through 3D printing, or additive manufacturing techniques. Macroscopic geometry and local topologies in scaffolds can be custom-designed by computational algorithms to control the permeability and mechanical properties [4]. In the study on seeding chondrocytes in scaffolds made through 3D fiber deposition and particle leaching, a significantly higher GAG content was observed in the fibrous scaffolds compared to the porous foams (Fig. 19.2A and B) [25]. Besides the fibrous morphology, the more uniform porous structure via controlled fiber deposition was suggested relevant to the better scaffold function.

In the recent decade, scaffolds have been fabricated toward mimicking the anisotropic natural tissue, which often contains pore size gradients, layered structures, and hierarchical pores at different length scales. Scaffolds showing gradient in pore size were generated through conventional fabrication methods or computational-aided prototyping [26,27]. For example, in the gradient scaffold fabricated through an additive manufacturing process and seeded with human MSCs, a gradual increase in chondrogenic markers was observed within the gradient structures with decreasing pore size (Fig. 19.2C) [26]. In other studies, scaffolds showing dual or multiple architectural features were also designed. For example, scaffolds were fabricated to contain both macroscale and microscale pores to serve respective functions to support cell attachment and promote nutrient permeation ([28−30]). As shown in a composite cardiac scaffold in Fig. 19.2D [29], the scaffold was fabricated to contain microchannels, an interface, and grids in different layers, and all structures were simultaneously fabricated with microscale pores to

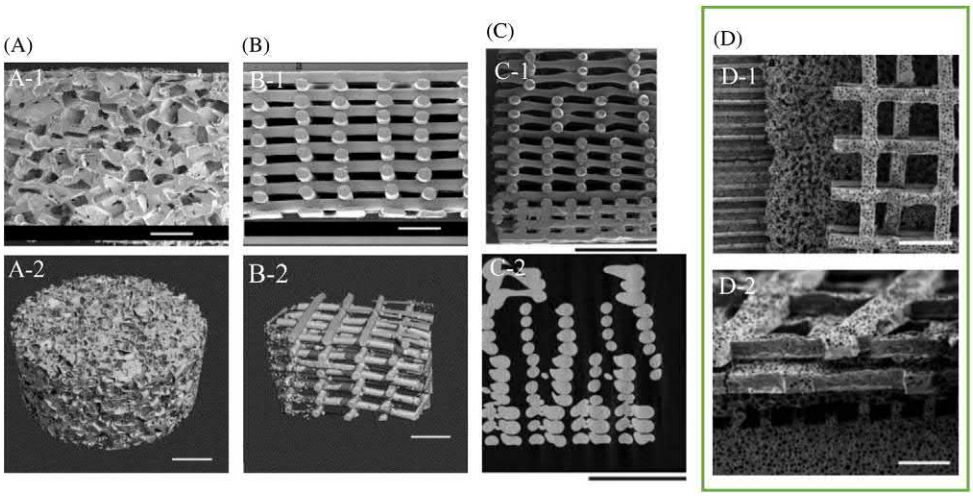

FIGURE 19.2 Evolving structures of porous scaffolds fabricated for cartilage tissue engineering: from **A** to **C**, scaffolds containing random pores, homogenous structures and graded structures. A-1, B-1 and C-1 are electron micrographs and A-2, B-2 and C-2 were reconstructed from μCT scans. (**A** and **B** were adapted and reproduced from *Malda, Woodfield et al. 2005* (scale bar, 1 mm), and **C** from *Luca, Szlazak et al. 2016* (scale bar, 2mm), with permission by Elsevier). **D)** two views of a multi-structure scaffold of hierarchical architecture containing porous channels, porous interface and porous grids for supporting vascularization and cardiac tissue engineering (adapted and reproduced from *Morgan, Sklaviadis et al. 2016* (scale bars, 500 μm (d-1) and 200 μm (d-2)), with permission by John Wiley & Sons).

promote the transport of molecules. These new trends in pore design highlight the needs for scaffolds with multifaceted and biomimetic functions in order to achieve optimal tissue growth.

Mechanics

The mechanical properties of the natural ECM are of paramount importance in dictating macroscopic tissue functions (e.g., bearing load) and regulating cellular behavior via mechanotransduction signaling. In designing tissue constructs, scaffold mechanical properties are often sought that resemble native tissue properties. Foremost, in the acute phase following implantation, the scaffold must fulfill the key mechanical functions of the tissue that is being replaced. For example, the earliest TE blood vessels based on cell-contracted collagen gels were not strong enough to withstand physiologic blood pressures and thus had to be reinforced by a tubular synthetic polymer mesh to ensure structural integrity [31]. TE blood vessels based on relatively strong nonwoven poly(glycolic acid) (PGA) scaffolds exhibited burst pressures exceeding physiological requirements upon implantation ($>$ 2000 mmHg) [32]. In addition to appropriately matching gross tissue mechanical properties, the scaffold must also provide an internal micromechanical environment conducive toward the de novo synthesis and organization of ECM. For example, while nonwoven PGA scaffolds have been successfully employed in blood vessel [32] and heart valve TE [33], their application to myocardial tissue has been comparatively challenging [34]. In contrast to the predominant load-bearing functions of vessels and valves, the primary function of myocardial tissue is cyclic contraction. While the out-of-plane compressive modulus of a typical nonwoven PGA scaffold is relatively low ($\sim 6.7 \pm 0.5$ kPa [35]), the in-plane tensile and compressive moduli resisting cardiomyocyte-mediated contraction are comparable at $\sim 284 \pm 34$ kPa [36].

The mechanical properties of TE scaffolds are determined in part by the bulk properties of their constituent materials (e.g., modulus of elasticity, degradation rate). For example, most hydrogel materials exhibit much lower strength and stiffness as compared to hydrophobic polyester materials. As traditional PLGA-based scaffolds have a limited subset of mechanical properties, new biodegradable materials have been developed, such as poly(hydroxyalkanoates) and poly(glycerol sebacate) to improve scaffold's toughness and elasticity [37,38]. Because of the high porosity and concomitant low material content, the mechanical properties of TE scaffolds are very often primarily dictated by the structural arrangement of their constituent materials (e.g., pore size, fiber diameter and orientation; Table 19.1) and associated modes of structural degeneration (e.g., fiber fragmentation, bond disruption). For example, the effective stiffness (E) (equivalent to initial tensile modulus) of nonwoven PGA scaffolds was predictably modulated by tuning the fiber diameter via NaOH-mediated hydrolysis [36]. Inhomogeneity is another important characteristic of natural tissues, which contain microdomains of distinct morphology and mechanical property to define tissue mechanics and mechanotransduction. The scaffolds generated containing

TABLE 19.1 Dependence of a scaffold mechanical property (initial tensile modulus) on the bulk material mechanical property and scaffold structure.

Material	Initial tensile modulus (MPa)		
	Fibrous scaffold	Foam scaffold	Bulk material
PGA	0.284 ± 0.034 (nonwoven) [36]	0.919 ± 0.067[a] (salt leach) [189]	18,780 ± 3430 (fiber) [36]
PEUU	8 ± 2 (electrospun) [190]	~1.4[b] (TIPS[c]) [191]	60 ± 10 (film) [190]
PGS	N/A	0.004052 ± 0.0013 (salt leach) [192]	0.282 ± 0.0250 (film) [193]

Several order-of-magnitude differences in modulus can be realized by starting with different bulk materials and/or by converting the bulk material into different porous scaffold structures (e.g., foam or fibrous). For comparison, the initial tensile modulus of a typical passive muscle tissue was reported to be 0.012 ± 0.004 MPa [45]. *PEUU*, Poly(ester urethane) urea; *PGA*, poly(glycolic acid); *PGS*, poly(glycerol sebacate).
[a]*Aggregate modulus obtained from creep indentation testing of PGA–PLLA scaffold.*
[b]*Estimated from the PEUU1020 stress–strain curve [191].*
[c]*Thermally induced phase separation.*

fibers and proteoglycan-rich microdomains provided a paradigm showing the mechanics of a scaffold could also be designed with microstructure- and domain-dependent properties [39].

In addition to the initial structure imparted during the fabrication of the TE scaffold, the dynamic degeneration, modulation, and alteration of mechanical properties need to be studied. For example, while 50:50 blend PGA/PLLA scaffolds do not undergo significant mechanical degeneration over a period of 3 weeks [40], scaffolds dip-coated with the biologically derived thermoplastic poly(4-hydroxybutyrate) (P4HB) incur rapid loss of rigidity with cyclic flexural mechanical loading as the P4HB bonds between fibers are disrupted [41]. Depending on the kinetics of scaffold hydrolysis, structural degeneration may be more pronounced and thus represents a more important consideration in scaffold design.

With the evolving tissue-formation process, the effective mechanical properties of scaffold will be determined by the combined effects of the cells, ECM, scaffold, and their unique micromechanical interactions. The appropriate formulation and validation of a mathematical model to simulate and/or predict the mechanical properties of a scaffold is critical to monitor the scaffold system and understand the dynamics in the tissue scaffolds. While standard phenomenological models are useful in characterizing the gross mechanical behavior of a scaffold or an engineered tissue construct (i.e., for meeting organ and tissue-level functional requirements), recent formulated structural-based models have been developed to investigate the micromechanical environment presented at the cell level. Structural-based models can either be computationally driven, as in the work of Hollister et al. [4,42], or purely analytical [36,43] to bridge the gap between the disparate length scales of cells, tissues, and scaffolds. For example, contrary to traditional rule of mixtures theories [44], higher order reinforcement effects were observed via the structural-based modeling of commercial nonwoven PGA and PLLA scaffolds as a result of the deposition of ECM molecules. The scaffolds were effectively stiffened due to extensive fiber–fiber crossover points that also imparted changes in fiber geometry and micromechanical properties, highlighting the importance of an accurate micromechanical representation of the TE scaffold [36,43].

The effects and associated mechanisms of how mechanical properties of the scaffolds instruct the cell behavior have also been studied using hydrogel matrices. To understand the role of material elasticity on cell behavior, myoblasts were cultured on collagen strips attached to glass or polymer gels of varied elasticity [45]. Cells were found to differentiate into a striated, contractile phenotype only on substrates within a very narrow range of muscle-like stiffness (i.e., 8–11 kPa) (Fig. 19.3A) [45]. In another study, it was further demonstrated that the matrix stiffness regulates stem cell differentiation independently of protein tethering and porosity [46]. More recent studies reported that the stiffness of hydrogel matrices generate essential microenvironmental cues to regulate stem cell behaviors, including phenotype, self-renewal, and differentiation, suggesting mechanical property as an important factor in driving the scaffold function [47–49]. In addition to stiffness, the viscoelastic property of hydrogel matrices was investigated, and it was found that in gels of faster relaxation, cell spreading/proliferation, osteogenic differentiation in MSCs, and cartilage matrix formation by chondrocytes could all be enhanced (Fig. 19.3B) [50,51]. The mechanical sensing and mechanotransduction that may involve cellular traction force and the stress-stiffening-mediated mechanism [52] highlight how the reciprocal remodeling process between cells and matrices should be considered in the design of scaffolds.

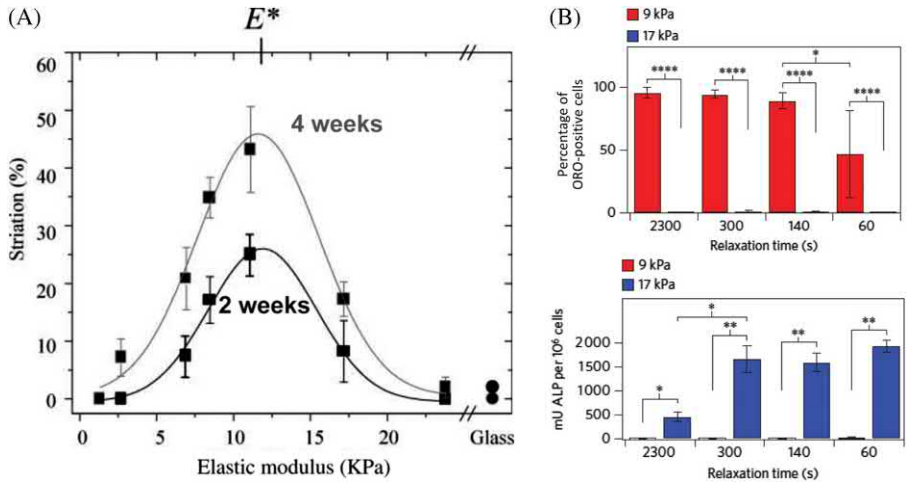

FIGURE 19.3 Cell differentiation exhibits sensitivity to local micromechanial properties of scaffolds. **A)** Substrate stiffness had a profound influence on myocyte differentiation, with optimal differentiation (as assessed by striation) occurring within a very narrow range of muscle-like stiffnesses (i.e. 8-11 kPa) (reproduced from *Engler, Griffin et al. 2004*, with permission by Rockefeller University Press). **B)** Quantification of the percentage of cells positive for oil red O (upper image) and a quantitative assay for alkaline phosphatase activity (lower image) showing MSCs increased osteogenic differentiation in gels with faster stress relaxation (reproduced from *Chaudhuri, Gu et al. 2016*, with permission by Nature Spring).

Because the foremost role of the scaffold following implantation is to temporarily fulfill the key mechanical functions of the replaced tissue, it is also essential to consider the physiological loading state of the native tissue and tissue remodeling of scaffolds from human recipient. While the physiological loading state may be highly complex, certain mechanical testing configurations are applicable. For example, the physiological loading state of a semilunar heart valve leaflet includes multiaxial flexural, tensile, and fluid shear stress components. In light of the strong planar anisotropy and tri-layered structures exhibited by native leaflet tissues, biaxial tensile testing [53] and flexural testing [54] have been employed to characterize their behavior. In another study of heart valve TE using PGA meshes, computational modeling based on mechanically induced tissue remodeling predicted leaflet compression and subsequent shortening as a result of physiological loading pressures [55]. More physiologically relevant geometry was designed to counteract the leaflet retraction, and the long-term functionality study demonstrated the potential of computational simulations to guide the design of TE constructs [56,57].

Electrical conductivity

Electrical conduction is an important mechanism that enables cellular signaling and function in many types of tissues. The cardiac electrical conduction system is essential to maintaining synchronous beats that pump blood in an ordered fashion. In the process of bone regeneration, naturally occurring piezoelectric properties of the apatite crystal are hypothesized to generate electric fields involved in bone remodeling. The nervous system possesses the well-known system of electrochemical signaling. Much research has been carried out using materials to record from and influence bioelectric fields. In making tissue scaffolds, electrically conductive biomaterials have been studied to understand their abilities to interface with bioelectrical fields in cells and tissues to replicate normal electrophysiology. Notably, most of these conductive scaffolds have been investigated for engineering and repair of cardiac and neural tissues.

A wide variety of electrically conductive polymers have been incorporated and developed into scaffolds, and they can be generally categorized into polymeric and carbon-based materials. Due to the ease of fabrication and the demonstrated biocompatibility, poly(pyrrole) (PPy), poly(aniline), and polythiophene derivatives are the most studied conductive polymers [58,59]. In contrast to static charge seen in conductive materials such as PPy, piezoelectric materials, including poly(vinylidene fluoride) and poly(tetrafluoroethylene), display transient charge in response to mechanical deformation [60]. There are also efforts to synthesize biodegradable conductive polymers, for example, through the incorporation of 3-substituted hydrolysable side groups [61], connecting degradable ester linkages with oligomers of pyrrole, aniline, and oligothiophene [58,62,63], and emulsion/precipitation of PPy in poly(D,L-lactide) [64]. In recent years, carbon-based nanomaterials, including carbon nanotubes and graphene, have emerged in biomedical studies due to their exceptional electrical conductivity [65]. While methods have been established to blend the carbons into traditional scaffolds or hydrogels, foams or fibrous scaffolds mainly based on carbon nanotube or graphene were also fabricated through methods such as vapor deposition and sacrificial template [66,67].

Electrical stimulation was found to promote cellular response and integration of cells dispersed in scaffolds. Neonatal cardiomyocytes cultured on collagen sponges

and matrigel show synchronized contraction in response to applied electric fields [68,69]. Electrical stimulation also promoted the maturation of stem cell–derived cardiomyocytes [70]. The beneficial effects of rendering the scaffold materials conductive were then often demonstrated in synergy with electrical stimulation in a variety of cells. In an early study, applied potentials were applied change surface properties of conductive polymers, resulting in altering the shape and function of bovine endothelial cells, including DNA synthesis and extension [71]. Later the electrical stimulation also promoted neurite outgrowth on the oxidized polypyrrole films [72]. More recently, human neural stem/progenitor cells were cultured in a 3D hydrogel construct containing nanocarbon tubes and PPy, and the incorporation of conductive materials and electrical stimulation upregulated the calcium channel expression and intracellular calcium influx in differentiated stem cells [73]. In another study the carbon-based 3D graphene foam promoted the differentiation of neural stem cells toward astrocytes and neurons and in the meantime showed good coupling with cells under electrical stimulation [67]. The carbon nanotube was also observed to act as electrical nanobridges between cardiomyocytes and promoted electrical coupling, synchronous beating, and cardiomyocyte function [74].

A number of conductive scaffolds have been investigated in vivo to evaluate their function to promote cardiac and neural tissue repair. For example, in the study on a PPy–chitosan hydrogel, the conductive material was found to improve the cardiac function following an myocardial infarction by accelerating transverse conduction velocities compared to nonconductive controls [75]. Besides the early polypyrrole-based neural prosthetics [76,77], conduits were recently fabricated containing single-layer or multilayer graphene, and the electrically conductive scaffolds exhibited the function to promote axonal regrowth and remyelination after the peripheral nerve injury [78].

Over the years, the mechanistic basis for each of these electrical–material–tissue interactions is still not fully understood and will continue to be an important area for future study. Some hypotheses can be found in the literature. This enhanced function of engineered cardiac tissues may be due to greater ultrastructural organization in response to electric fields [68]. Increased neurite outgrowth with electrical stimulation may be caused by better ECM protein adsorption [79] rather than direct effects on the cell itself although there is ample evidence of the latter. Electrophoretic redistribution of cell surface receptors likely governs the galvanotropic response of neurons to a horizontally oriented two-dimensional (2D) applied field [80]. Such mechanisms do not fully explain altered cell function in response to stimulation applied to the substrate or material relative to the medium or a distant ground. For such depolarization, signaling through voltage-gated calcium channels can activate ubiquitous second messengers such as cyclic adenosine monophosphate (cAMP) and alter gene transcription affecting learning, memory, survival, and growth [81]. Such secreted gene products might be used to enhance survival of host cells surrounding an implanted scaffold. Lately, in the study in which electrical preconditioning of human neural progenitor cells on PPy scaffolds enhanced the stroke recovery, it was shown that the electrical stimulation may have improved the functional outcome through regulating the angiogenesis-relevant vascular endothelial growth factor-A (VEGF-A) and hypoxia-inducible factor-1α (HIF-1α) pathways [82]. Given that applied electric fields can so profoundly affect the cell function, conductive 3D scaffolds will be important tools for harnessing this interaction to create functional tissues.

Surface properties

Cells interact with scaffolds primarily through the material surface, which is dominated by the surface chemical and topological features. The surface chemistry here refers to the insoluble chemical environment that the scaffold surface presents to cells, which can be directly from the biochemical compositions of the bulk, and/or the substances derived from surface adsorption or chemical reactions. Besides mediating cell behavior and functions inside scaffolds, controlled surface properties are of central importance in directing the inflammatory and immunological response. Controlled surface properties may be useful for ameliorating the foreign body reaction at the host–scaffold interface in vivo [83,84].

Surface chemistry

Each type of synthetic, natural, or composite scaffold gives rise to a set of distinct surface chemical characteristics governed by the material chemistry and its physical form (such as crystallinity, charge, and topology). Although numerous efforts have been made to tailor the scaffold surface, the chemical environment can exhibit extremely complicated patterns within the biological milieu. Complex processes such as the spontaneous adsorption of a diversity of proteins from biological fluids to the scaffold surface, and the protein surface conformation are difficult to analyze, which however exert profound effects on the scaffold performance. To tailor the scaffold chemical properties, the interactions of scaffolds with different environmental factors need to be considered.

Scaffolds derived from natural ECM materials such as collagen, fibrin, hyaluronic acid (HA), proteoglycans, or their composites have the advantage of directly containing

innate biological ligands that cells can recognize and provide natural mechanisms for tissue remodeling. ECM analogs have been created to emulate an appropriate tissue regeneration environment. For example, as an essential ECM component in natural cartilage tissue, collagen type II scaffolds may have better biochemical properties to maintain chondrocyte phenotype and enhance the biosynthesis of GAGs compared to collagen type I [85]. Fibrin, the native provisional matrix of blood clots, can provide ligands to initiate cell attachment and ECM remodeling [86]. HA plays a role in morphogenesis, inflammation, and wound repair, and the cell−ECM interactions mediated through receptors such as CD44 and the receptor for hyaluronan mediated motility (RHAMM) can be activated in scaffolds with HA constituents [86]. Recently, scaffolds directly derived from decellularized ECM have attracted great interests due to the possibility to preserve and recapitulate the native tissue components and functions. Decellularized ECM can be processed directly into various forms, including patches, scaffolds, powders as well as hydrogels, and has provided a versatile platform for TE applications [87−89]. The tissue regenerative functions of decellularized matrices are associated with mechanisms involving modulation of the immune response, release or exposure of growth factors and cryptic peptides, and recruitment of progenitor cells [88]. Recently, arrays of 2D and 3D tissue ECM substrates were fabricated and the tissue-specific cellular responses were studied [90]. The methodology may be useful for understanding the microenvironment and function of natural ECM-based materials.

Synthetic scaffolds offer a variety of mechanisms to modulate cell behavior. Chemical reactions with biological fluid remodel the scaffold surface and affect tissue growth through both reaction dynamics and kinetics. Studies have shown that bioactive glasses (Class A Bioglass composed of 45%−52% SiO_2, 20%−24% CaO, 20%−24% Na_2O, and 6% P_2O_5) have superior osteoproductive properties than either bioactive hydroxyapatite or bioinert metals and plastics. The difference was found to be due to the surface reaction kinetics in physiological fluid. The rapid reaction rate that converts amorphous silicate to polycrystalline hydroxyl-carbonate apatite on the bioglass surface is the key to positively regulating the cell cycle and bone formation [91]. Physical processes also play active roles in controlling the material−cell interface. Because of the adsorbed protein moieties from serum or body fluid, many polyester-based scaffolds, such as those made from poly(α-hydoxy esters), exhibit adequate adhesion to support cell attachment and tissue growth in some in vitro and in vivo applications. Methods that alter the surface hydrophobicity, for example, by changing monomer compositions or by chemical surface treatment, can potentially improve the scaffold performance [92−94]. For example, biodegradable foams of hydrophobic polymers (e.g., PLLA and PLGA) can be efficiently wet by two-step immersion in ethanol and water. This surface treatment could overcome the hindered entry of water into air-filled pores to facilitate cell seeding [92].

Surface modifications of scaffolds have been developed to generate surface chemical specificity and recognition. The surface chemistry can be created by either incorporating bioactive moieties directly in the scaffold bulk or modifying the surface. These moieties bound to scaffolds trigger desired specific intracellular signaling. In particular, many synthetic and natural hydrogel materials [e.g., poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), alginate, and dextran] are protein-repellent, and immobilizing biomolecules to such hydrogel scaffolds may be especially useful in tailoring the surface chemistry for cell−material interactions at the molecular level.

Cell adhesion mediated through extracellular adhesive proteins is involved in many intracellular signaling pathways that regulate most fundamental cell behaviors, including differentiation, proliferation, and migration. Enriching scaffold surfaces with specific ECM-derived adhesion proteins has been widely applied to scaffold modification. PLGA-based scaffolds have been coated with fibronectin by physical adsorption for supporting growth and differentiation of human embryonic stem cells in 3D [95]. Fibronectin was covalently attached to PVA hydrogels for improved cell adhesion, proliferation, and migration [96]. Fibrinogen was also denatured and fused into the backbone of a PEG hydrogel material [97] to elicit cellular responses. Elucidating the underlying molecular mechanisms on scaffold surface however is not a trivial task. Fundamental studies have been carried out to understand how the adsorption and denaturing of proteins can lead to different cellular responses at the material surface and may provide a molecular basis to control cell−material interaction and specificity for rational scaffold design [84].

Immobilizing peptide ligands derived from the active domains of ECM adhesion proteins to scaffolds is another major approach to generate specific surface-bound biological signals. For example, integrins, the principal adhesion receptor mediating cell−ECM attachment, comprise a family of more than 20 subtypes of heterodimeric transmembrane proteins. Each of them recognizes and interacts with certain types of ECM adhesion proteins to activate a cascade of signaling pathways to regulate essential cell activities and functions [98]. Integrins can be activated by short peptides in similar ways, for example, arginine-glycine-aspartate (RGD) from fibronectin and tyrosin−isoleucine−glycine−serine−arginine (YIGSR) from laminin. As these short peptides are relatively stable and economical to use, incorporating them into scaffolds

represents an important way to generate surface biomimicry and has been widely investigated for TE scaffolds [99–102]. Recent studies were also carried out to improve the specificity of RGD-based sequence to bind and activate integrin subtypes. Recombinant fragments derived from fibronectin were shown to enhance the osteogenic differentiation of MSCs through specifically engaging the $\alpha_5\beta_1$ integrin versus $\alpha_v\beta_3$ [103]. The importance to improve the molecular specificity was also demonstrated in the experiment comparing the ligands specifically binding to $\alpha_3/\alpha_5\beta_1$ and $\alpha_v\beta_3$, in which the $\alpha_3/\alpha_5\beta_1$-specific scaffolds showed the superior function to modulate the vascular patterning and support the formation of mature and nonleaky vasculature [104].

Like most biomolecules in natural ECM, the functions of immobilized bioactive ligands are influenced by their spatial characteristics in modulating membrane receptors and activating intracellular signaling. In modified fibrin gels, 3D neurite migration demonstrated a biphasic dependence on the RGD concentration, with the intermediate adhesion site densities (between 0.2 and 1.7 mol of peptide/mol of fibrinogen) yielding maximal neurite extension compared with the higher densities, which inhibited the neurite outgrowth [105]. In another study, integrin clustering, a prerequisite to many integrin-mediated signaling pathways, was recapitulated by the RGD nanoclusters immobilized on a comb-polymer substrate [106]. More studies through nanotechnologies identified the spacing requirement of RGD ligands at 70 nm on 2D surface to trigger cellular response [107,108]. In solutions the clustering of adhesive ligands was also investigated through dendrimer constructs for the purpose of promoting cellular response [109].

Surface topography

The material–cell interactions mediated through topographical features have traditionally been studied through planar substrates. Surface modification techniques, including photolithography, contact printing, and chemical treatments, have been developed to generate micro- and nanoscale surface topographical features. Surface topographical features such as ridges, steps, and grooves were found to guide cytoskeletal assembly and cell orientation. Compared to smooth surfaces, surfaces with textures such as nodes, pores, or random patterns are often associated with marked changes of cell morphology, cell activities, and the production of autocrine/paracrine regulatory factors [110,111]. In general, surface roughness increases cell adhesion, migration, and the production of ECM. Cells sense and respond to topographical features in a scale-dependent way [112]. As demonstrated on titanium surfaces, while microtextures increased osteoblast attachment and growth, only the presence of nanoscale roughness led to enhanced cell differentiation in connection with elevated growth-factor production [113].

Current fabrication techniques can be used to generate a wide variety of topographical features in scaffolds. Scaffolds can be randomly packed with regular or irregular geometries and shapes (e.g., particles, pellets, and fibers), or condensed with amorphous structures (e.g., foam and sponge), or fabricated with specifically designed architectures. Surface topography can also be generated and varied through coating nanoparticles of different shapes onto scaffolds [114]. Depending on the topographical feature size and the scaffold geometry, scaffolds provide a variety of topographical properties at different length scales. In 3D scaffolds, cells may dwell on or be surrounded by curved surfaces provided by fibers or walls of pores [110]. The topography may pertain to the gross contact area or subcellular interface between cell and scaffold, generating different cell responses that must be correlated with a specific scale and cell–scaffold interaction. The cellular responses can be investigated at multicellular, cellular, and molecular levels. In the studies on MSCs seeded onto polymeric fabrics containing microscale fibers, increased fiber diameter favored cell attachment and mature cell adhesion and promoted the MSC differentiation into osteoblast-like cells, with the optimal fiber diameter found at 9 μm [115]. Mechanistically, it was found that the fibrous topography triggered RhoA/ROCKII signaling in concert with Myosin IIa activation, which accounted for subsequent cell tension and osteogenic differentiation of osteoprogenitors grown on poly(methyl methacrylate) fibers [116]. In another study the electrospun fibrous scaffolds also enhanced the paracrine function of MSCs compared to the planar substrate and benefited skin tissue repair as a result of the upregulated expression of proangiogenesis and immunomodulatory factors [117].

The size scale of most natural ECM components, for example, fibrous elastin and collagen, falls into the range of several to tens of nanometers. The extracellular environment is dominated by nanoscale topographical features, such as nanopores, ridges, fibers, ligand clusters, and high surface area-to-volume ratios in 3D. Such native topographies can be recapitulated to a degree in scaffolds made of natural ECM polymers such as collagen and elastin. As synthetic materials have the advantage of greater control over scaffold properties, interest is growing in developing techniques to generate nanoscale topography. Surface treatment techniques such as sodium hydroxide etching have been used to generate nanoscale roughness to increase cell adhesion, growth, and ECM production [118]. 3D PLLA-based scaffolds containing nanofibers have been produced by thermally induced phase separation processes [119,120], and selective surface adsorption of adhesion proteins was observed. In a more versatile method, electrospinning techniques have been used to

fabricate a variety of synthetic and natural materials with different hydrophobicities into fibers with diameters ranging from a few to hundreds of nanometers [121]. Another important approach involves building scaffolds from the bottom up. Polypeptides made of 12−16 amino acids have been designed to form hydrogel scaffolds through β-sheet assembling [122]. Amphiphilic molecules consisting of a hydrophilic peptide head and a hydrophobic alkyl tail self-assemble into nanocylinders to form interwoven scaffolds [123,124].

Nanofibrous scaffolds have demonstrated abilities to support nanoscale-specific cell activities. For example, on TiO_2 nanotubes, human MSCs demonstrated adhesion or differentiation into osteoblast-like cells with the tube diameter at 30 or 70−100 nm, respectively [125]. When cardiomyocytes were cultured in meshes made of electrospun poly(ε-caprolactone) nanofibers, they expressed cardiac-specific markers and were contractile in 3D scaffolds [126]. In a scaffold based on self-assembled peptide amphiphilic molecules containing the laminin epitope isoleucine-lysine-valine-alanine-valine (IKVAV), neural progenitor cells selectively differentiated into neurons [123]. Some possible mechanisms associated with nanotopography in scaffolds may be related to cell receptor regulation (clustering, density, and ligand-binding affinity) on nanofibers, nutrient gradients in nanoporous matrices, mechanotransduction induced by the unique matrix mechanics, and the conformation of adhered proteins for cellular recognition sites. As it is likely that topographical cues are in close relationship with cell stiffness and adhesion, the mechanical properties of scaffolds should be considered as an important parameter in conjunction with topography to understand the combinatorial effects of scaffolds on triggering specific cell response.

Temporal control

Scaffold degradation

Unlike permanent or slowly degrading implants that may serve to augment or replace organ function (e.g., hip implants, artificial hearts, or craniofacial plates), TE scaffolds serve as temporary devices to facilitate tissue healing and regeneration process. The regeneration of a fully functional tissue ideally coincides in time with complete scaffold degradation and resorption. Controlling degradation mechanisms allows scaffolds to temporally cooperate with cell and tissue events via changes in scaffold properties and functions. Tuning the scaffold degradation rate to make it kinetically match with the evolving environment during tissue healing and regeneration is an important design criterion.

During scaffold degradation, some scaffold properties and functions may weaken or diminish with time. In general, there exist lower and upper limits on the optimal degradation rate that may vary with different cellular or tissue processes, scaffold chemical compositions, and scaffold functions. For example, scaffolds often need to serve mechanical functions, such as in bone implants to support compressive loading while maintaining an environment permissive to new bone formation. If a material degrades prior to transferring mechanical load to the new tissue, the therapy would fail [127]. Alternatively, materials in bone implants that degrade too slowly may cause stress shielding, thereby impeding the regeneration process and potentially endangering surrounding tissues [128]. In skin wound models, healing can be compromised when the scaffold degradation occurs too quickly, whereas scar tissue occurs when the degradation is too slow [19]. The optimal skin synthesis and prevention of scar formation could be achieved when the template was replaced by new tissue in a synchronous way, that is, the time constant for scaffold degradation (t_d) and the time constant for new tissue synthesis during wound healing (t_h) were approximately equal [19]. Matching tissue formation with material degradation thus requires coupling of specific temporal aspects of tissue-formation processes with chemical properties of the scaffold.

Scaffold degradation can occur through mechanisms that involve physical or chemical processes, and/or biolological processes that are mediated by biological agents such as enzymes in tissue remodeling. Degradation results in scaffold dismantling and material dissolution/resorption through the scaffold bulk and/or surface. In the passive degradation mode the degradation is often triggered by reactions that cleave the polymer backbone or cross-links within the polymer network. Many polyester scaffolds made of lactic acid and glycolic acid, for example, PLLA and PLGA, undergo bulk backbone degradation due to their wettability and water penetration through the surface. Hydrophilic scaffolds such as hydrogels made of natural or synthetic materials cross-linked by hydrolysable bonds (e.g., ester, carbonate, or hydrazone bonds) also convert to soluble degradation products predominantly through the bulk [129,130]. Chemical degradation can be conveniently varied through scaffold physical and chemical properties such as the backbone hydrophobicity, crystallinity, glass transition temperature, and cross-link density. Because of this flexibility, the degradation rate can principally be engineered for optimal tissue regeneration [127,131].

Scaffolds degrading through passive mechanisms exhibit limited capabilities to match with tissue growth and wound healing. In bulk degradation the accumulation of degradation products may exert adverse effects on tissue, for example, acidic products from PLGA degradation. It is also difficult to tailor the degradation to match the healing rate, which may vary with wound conditions

such as age of the patient, severity of the defect, and presence of other diseases. In order to exert more control over the degradation properties of a scaffold and to attempt to tailor the degradation of the scaffolds, consideration of pertinent wound healing and tissue regeneration mechanisms is required. For example, would healing is a highly complex, yet orchestrated cascade of events controlled by a vast array of cytokines and growth factors that generally involves three phases, including inflammation, granulation tissue formation, and remodeling of the ECM. Scaffolds should be designed to degrade in vivo during the formation of granulation tissue and/or during the remodeling process. Ideally these materials should withstand uncontrolled dissolution or degradation at physiologic conditions while being resorbed by natural cell−mediated processes. Many inorganic scaffolds for bone TE demonstrate biodegradable and bioresorbable characteristics to facilitate new tissue formation [132−134].

While scaffolds derived from naturally derived materials such as collagen and decellularized matrix are capable of incurring biological degradation, protease-mediated degradation mechanism can be designed in scaffolds so that the scaffolds are dismantled through cellular proteolytic activities [135,136]. In one of the early studies a PEG hydrogel modified with adhesion ligands was cross-linked with molecules containing matrix metalloproteinase (MMP) peptide substrates. Human primary fibroblasts inside the gel migrated inside the gel matrix via secreting MMP to break down the polymer chain. The protease-degradable materials allow cells to remodel the surrounding environment, providing an important biomimetic strategy to design scaffolds with controlled degradable mechanism. The MMP-sensitive hydrogels have been investigated for different tissue regeneration scenarios such as bone regeneration and cardiac differentiation by supporting 3D cell culture and cell invasion in vivo [137]. The MMP-sensitive substrates were also later introduced into a number of hydrogel-based scaffolds, including polysaccharides networks [138,139], self-assembling peptides [140,141], and the electrospinning fibrous structure [142]. The approach demonstrated how scaffold degradation can be engineered to synchronize with wound healing and new tissue synthesis via natural mechanisms. MMP-sensitive matrices have not only been tested in vivo applications but also used to discover degradation-associated cell−matrix interactions [143−145]. In particular, degradation was found to be a parameter separated from matrix mechanics and mediated the cellular traction in MSCs to drive osteogenesis, providing an important insight into how cell would sense and respond to microenvironment cues from TE scaffolds [144].

Delivery of soluble bioactive factors

The incorporation of delivery systems in 3D scaffolds offers an indispensable platform for enabling temporal and spatial control in tissue constructs. The release of bioactive factors can provide direct instructive and regenerative signals while limiting side effects at unwanted body locations [146]. Although numerous material-based controlled release systems are at the disposal of tissue engineers, most of them need to be adapted when applied to tissue scaffolds. The release of soluble factors from scaffolds can be mediated through single or multiple mechanisms, for example, by diffusion, dissolution, scaffold or carrier degradation, or external stimuli. In particular, delivery of growth factors has been studied for various tissues, due to their important roles in instructing cell behavior.

Built upon established particle-based delivery systems, one common method for controlling the release from a scaffold involves prefabricating biofactor-loaded particles and embedding them into a scaffold matrix [147,148]. The release of biofactors in these systems can be delayed with a minimized burst effect, compared to the systems made of particles alone. Typical particle carriers include PLGA and hydrogel microspheres. This method takes advantage of established systems but involves composite matrices that influence the release profile. Alternatively, soluble factors may be incorporated directly into the scaffold itself without a secondary carrier/matrix. This often requires the scaffold to be fabricated under mild physiological conditions to preserve the bioactivity of proteins or other biofactors. Growth factors and proteins have been incorporated in scaffolds through surface coating [149], emulsion-freezing drying [150], gas-foaming/particulate leaching [151,152], and nanofiber electrospinning [121,153]. Different delivery profiles of growth factors or DNA plasmids were achieved. Fibrous scaffolds obtained via electrospinning were investigated widely in recent years. The release function can be introduced into the synthetic and natural electrospun fibers via surface processing such as adsorption, immobilization of particles, and conjugation of biofactors. Coaxial electrospinning process can give core−shell structures, which allow encapsulation of biomolecules and deliver them with reduced burst effect and prolonged duration [154]. Due to their hydrophilic and biocompatible nature, hydrogel scaffolds are amenable to incorporating proteins and plasmid DNA, yielding both higher loading efficiencies and bioactivity compared to PLGA-based materials. Biofactors have been immobilized in hydrogel matrices via physical interactions and/or covalent chemical bonds for prolonging retention time and controlling release via designed mechanisms [155,156]. Heparin has in particular been used in hydrogels to bind to growth factors and sustain

their release and inspired the design of other types of affinity-based systems, which have also been developed [157].

Scaffolds that integrate controlled release methods have been used in conjunction with scaffolds for a variety of purposes, including enhancing tissue formation, stimulating angiogenesis, guiding cell differentiation, and facilitating wound healing [156,158]. Delivering growth factors from scaffolds has demonstrated advantages over using the free form directly [159]. Synergistic effects on accelerating tissue regeneration have been observed when scaffolds, cells, and growth factors are combined. For example, autologous bone marrow–derived cells transplanted with scaffolds containing bone morphogenetic protein-7 resulted in the greatest bone formation compared to constructs without either growth factor or cells [160].

A major challenge in delivering biofactors involves achieving meaningful pharmacokinetic delivery. The dosage, release kinetics, and duration time should be optimized and tailored to tissue growth/healing processes. For example, the uncontrolled release of BMP-2 may have led to unwanted outcome of Infuse, which is used clinically to promote bone repair. Scaffolds have been investigated toward releasing a low dose of BMP-2 [161]. Scaffolds capable of biomimetic delivering of biological factors also provide attractive strategies in TE. Many growth factors are tightly sequestered in the ECM as inactive precursors and get released through the interaction with cells via specific protease-mediated mechanisms. Based on affinity or protease-sensitive mechanisms, hydrogel matrices containing immobilized growth factors allowed for the release of growth factors through plasmin activity [162,163].

Sequential delivery adds a new layer of temporal control in scaffolds integrated with release functions and has been investigated for angiogenesis, cardiac tissue, bone engineering/regeneration, etc. [146]. In one study, to simulate the temporal pattern of VEGF and platelet-derived growth factor (PDGF) in angiogenesis, PLGA-based scaffolds have been developed to deliver these two angiogenic factors with distinctive kinetics for rapid formation of a mature vascular network [164]. In the study on knee meniscus repair, nanofibrous scaffolds were designed to first release collagenase to generate ECM porosity and then PDGF to attract angiogenic cells, showing a delivery paradigm of temporal and functional coordination [165].

Spatial control

The hierarchically ordered organization of cells and ECM in 2D and 3D spaces is a basic yet most important characteristic of tissue structure. The ordered structures at meso- and microscales give rise to specific geometry and function of tissue and organ and are prerequisites to provide signals to guide cell activities, including localization, alignment, and formation of conjunction to induce tissue morphogenesis in 3D space. Spatial control in 3D scaffold therefore not only pertains to generation of anatomically relevant structures but also involves manipulation of appropriate spatial cues at multicellular levels to enable ordered cellular arrangement and tissue formation.

Recapitulation of the spatial characteristics of a natural tissue in scaffolds is a process dependent on the choice of materials and fabrication techniques. The mechanical, physical, and chemical properties determine how the material should be processed, while different material modification and fabrication methods may configure the shape and microstructure at a certain length scale or have precision limitation. The use of conduit scaffolds has been investigated widely for promoting nerve tissue repair or replacing small-diameter blood vessel. For example, PLGA conduit devices facilitated and guided Schwann cell attachment for peripheral nerve regeneration [166] and multiple-channel, biodegradable scaffolds were fabricated to transplant Schwann cells and guide directional growth of axons for treating spinal cord injury [167] Similar studies illustrate how defined shape and void spaces in scaffolds can provide spatial guidance in vitro or at the defect site to define cell localization/distribution to promote tissue growth. However, many tissues contain complex anatomical and microarchitecture that would be hard to replicate through one fabrication process such as molding or templated synthesis. Recent investigations therefore have been focused on how to improve the processability/shape fidelity of materials and versatility/precision of fabrication methods, so that scaffolds can be generated to contain 3D microanatomical structures and support functions with physiological relevance.

In terms of spatial cues, anisotropic characteristics of pore architectures, mechanics, surface properties, degradation, and delivery can all potentially generate signals recapitulating haptotaxis and chemotaxis for guided tissue growth. The most studied approaches in particular involve topographical structures and chemical patterning to provide contact guidance. Planar materials/substrates have been developed containing aligned longitudinal regions such as ridges, grooves, wrinkles, and chemical heterogeneities to induce cell orientation and organized aggregation [110,168]. As to applications, cells with geometrical patterns obtained from 2D or "2.5D" topographical systems may be used directly for transplantation. For example, in the study of endothelial cell cords molded from microgooves, the strand-like microtissues could also define neovessel architecture *in vivo* and improve angiogenesis [169]. In another study, the human umbilical vein endothelial cells and human mesenchymal stem cells patterned on a polyester urethane urea were investigated as a

cardiac patch that outperformed the control patch without cellular patterns [170]. Nonetheless, given the numerous studies conducted on 2D systems, accelerating the translation of spatial guidance and geometric control of cells to 3D is of great importance.

To induce cell alignments in 3D, orientation of fibers was generated in scaffolds. Collagen and fibrin aligned using magnetic fields [171,172] increased the rate and depth of axonal elongation in vitro and improved sciatic nerve regeneration in vivo as compared to scaffolds with random fiber orientations [171]. In the electrospun scaffolds, two layers of orthogonally oriented nanofibers made of poly(3-caprolactone) was subcutaneously implanted. The cell alignment inside the scaffold was found to be maintained by the oriented fibers, exhibiting histological similarity to intestinal circular and longitudinal smooth muscle [173]. More complex 3D structures may be created through templated synthesis and was effective to orchestrate the assembly of multiple cell types. This was shown in the study in which hydrogel matrices were fabricated to contain interconnected microchannels lined with endothelium as well as the interstitial zone with matrix and other types of cells [174]. 3D structure and anisotropy can also be created through stacking of thin sheets/laminates [175–178]. In particular, the recent techniques allowed the tissues assembled on 2D to be constructed to further build anisotropic architectures to combine cells and tissue components. Direct printing of materials with cells opened another important avenue to generate tissues with heterogeneous architecture. Different 3D printing processes demonstrated well-defined cellular patterns with high resolution, but the effects of spatial guidance on tissue morphogenesis in the holistic construction remain to be understood [179,180].

Due to the wide application of hydrogel materials in TE, creating heterogeneous chemistry in hydrogel matrices is an important approach to achieve spatial control for tissue guidance. Patterned photoimmobilization of adhesive RGD ligands was first demonstrated in the agarose matrix [181]. The patterns of adhesive and nonadhesive regions induced oriented axonal elongation from dorsal root ganglion cell aggregates in vitro. By developing photocleavage and photocaging chemistry in hydrogels, temporospatial control was further achieved through lithographical techniques and allowed for the generation of topographical and biochemical patterning with high precision and resolution [182–184]. Studies have also been carried out to incorporate chemical gradients in scaffolds. Gradients of proteins play important roles in tissue formation/remodeling during embryogenesis and wound healing. Concentration gradients of peptides and proteins in hydrogels were generated for the study of cell and tissue guidance. For example, the entrapment of nerve growth factor in a poly(2-hydroxyethyl methacrylate) hydrogel induced directional axonal growth from PC12 cells in vitro [185]. The gradient of VEGF-165 guided the migration of endothelial cells into the porous collagen scaffolds while showed no influence on the cell proliferation [186]. In a modified electrospinning process through dual syringes with varied velocities, the gradient of adhesive RGD peptides facilitated the cell infiltration into the fibrous HA scaffold [187]. Recently, combinatorial hydrogels were fabricated with gradient peptides that mimic cell–cell or cell–matrix interactions and biochemical formulations were screened in a high-throughput way for optimal chondrogenesis conditions [188]. Hydrogels providing patterned and tailored physical/chemical microenvironment may find broad applications in understanding cell–matrix interactions to advance the scaffold design.

Conclusion

Major challenges in 3D scaffold design involve the systematic understanding of how fundamental scaffold properties are interrelated in affecting cell behavior, and how these properties can be rationally programed—spatially and temporally—to provide the necessary signals to aid tissue formation/regeneration. Advanced fabrication methods and 3D scaffolds became available in the past to control the microenvironment with precision and resolution to study cell and tissue responses. New mechanisms and functions of 3D scaffolds were discovered and delineated, laying foundations for designing the new generation of TE scaffolds.

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Part Five

Transplantation of engineered cells and tissues

Chapter 20

Targeting the host immune response for tissue engineering and regenerative medicine applications

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Introduction

The field of tissue engineering/regenerative medicine (TE/RM) evolved as an alternative to conventional methods for the functional replacement and repair of injured or missing tissues and organs. While the field is no longer in its infancy and significant progress has been made, the task of building new tissue is obviously nontrivial. In addition to providing or recruiting stem/progenitor cells, and providing for an adequate vascular supply, the role of the immune system in tissue development and maintenance and the host immune response to TE/RM interventions is now recognized as critical for clinical success.

The typical approach of avoiding the immune response to an implanted biomaterial and/or TE/RM construct through either immunosuppression or development of "inert" biomaterials has been marginally successful. Insights derived from developmental biology and immunology, among other fields, have clearly shown the critical role of the immune response in the reconstruction of functional tissue, and that the constructive and regulatory signals of the immune system should be harnessed and embraced rather than avoided and suppressed. The present chapter will review specialized immune cell function in the context of tissue repair, how such cell types accomplish these feats, and novel TE/RM strategies that are now positioned as immune-stimulatory therapies rather than immune-avoidant therapies through purposeful targeting of the immune response.

Immune cells and their roles in building tissues after injury

Early attempts to engineer new tissues typically involved implantation of biomaterials, cells, or biomaterial-cell composites that avoided what was then considered to be the primary barrier to success: a foreign body reaction (FBR) [1]. With any implanted foreign object a series of immune cell actions leads to distinctive host response. In brief, recognition of a foreign body consists of adsorption of proteins on the implant surface, acute inflammation followed by chronic inflammation at the implant site, macrophage infiltration and frustrated phagocytosis with the formation of multinucleate foreign body giant cells, and eventual fibrous connective tissue within and/or around the implant. As a result, there is a barrier to the restitution of functional, innervated, and vascularized site-specific tissue [2,3].

Characterization of each of the different immune cell types and their signaling mechanisms that mediate the above processes is prerequisite to the identification of pivotal moments at which the immune response can be modulated and therefore contribute to improved outcomes. However, the complexity of such immune cell activities is significant. Any immune response, let alone a response to biomaterials or cell-biomaterial constructs, inevitably involves coordinated networks of signaling molecules combined with specific spatiotemporal roles and both direct and indirect signaling mechanisms by each immune

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cell type. An attempt to briefly summarize the known contributions to date of different immune cells to implanted materials and how they impact outcomes is described herein.

Neutrophils

Identified as the most rapid responders, neutrophils infiltrate the implant site and play a major role in phagocytosis of bacteria or cell debris. These cells dominate in number between minutes and days after implantation of the biomaterial or TE construct. Though much of the focus in the field to date has been upon other, less transient cell types, neutrophils play a clear role in the divergent immune responses commonly seen between synthetic versus biologic materials. For example, synthetic materials are associated with increased neutrophil recruitment compared to biologic materials, and this may be linked to disparate long-term outcomes [4]. Neutrophils themselves have been characterized as plastic cell types, with the ability to participate in both proinflammatory and antiinflammatory activities including generation of reactive oxygen species, in protease release, and the secretion of chemokines and cytokines that selectively recruit reparative macrophages allowing for matrix deposition and angiogenesis [5]. Recent work, however, has shown that selective depletion of neutrophils after synthetic versus biologic material implantation modulates myeloid cell phenotype and number. Neutrophil depletion did not have a significant impact upon the host response to biologic or synthetic materials, suggesting that neutrophil activity may be associated with, but not a causative player in FBRs to implanted constructs [4].

Eosinophils

The identified role of eosinophils in TE/RM outcomes has likewise been considered associative rather than causative in the FBR or tissue-integration outcomes. However, an emergent role for eosinophils in normal wound healing and in response to injury has prompted a more in-depth investigation into their role in biomaterial-mediated tissue remodeling. In muscle injury, for example, eosinophils are rapidly recruited to the injury site in large numbers. These cells secrete IL-4 during degranulation, which facilitates a regenerative response to acute muscle injury. Moreover, recent work using eosinophil-deficient mice has demonstrated that eosinophils are not only associated with but also are required for muscle regeneration as they are responsible for the majority of the IL-4 secreted at the muscle injury site [6]. Eosinophils also play a critical role in mediating cross-talk between other immune cells of both the innate and adaptive systems [7,8]. The emergent role of eosinophils in propagating the seemingly disparate processes of allergic reactions and maintenance of immune homeostasis makes them potentially attractive targets for immunotherapy and immunomodulatory biomaterials.

Macrophages

Macrophages have been the primary focus of TE/RM researchers due to their relative abundance in the early days following implantation of a biomaterial or cell construct. Findings of the past 20 years have dramatically changed the understanding of macrophage biology. Macrophages, such as T cells, can exist as either a proinflammatory, type 1 (M1), or antiinflammatory, type 2 (M2) cell based upon their differential metabolism of L-arginine [9], resulting in either nitric oxide (NO) generation or arginase generation, respectively. These metabolic profiles are associated with either proinflammatory or proremodeling effects. These two phenotypes play an essential role in normal wound healing [10], development [11], and tissue homeostasis [12]; physiological roles that implicate their potential as a target for RM therapies. Moreover, the ability of exogenously delivered stem/progenitor cells to exert beneficial in vivo effects may be due in part to their proclivity to promote immunomodulation that facilitates downstream constructive tissue remodeling [13–15]. In the context of implanted biomaterials an early macrophage transition from an M1-like phenotype to an M2-like phenotype has been shown to be predictive of host tissue integration at later time points [16]. Macrophages and their ability to contribute to a microenvironment that is either prohibitive or beneficial for tissue remodeling are now thought of as important determinants of TE outcomes, rather than merely antigen-presenting phagocytes.

Dendritic cells

Dendritic cells have not been considered as primary determinants of success or failure of a biomaterial in clinical applications. However, dendritic cells directly interact with implanted constructs and play a significant role in facilitating proregenerative cross-talk among other immune cells, namely between macrophages and cells of the adaptive immune system. For example, dendritic cells can respond directly to biomaterial-associated molecules such as extracellular matrix (ECM) components and the degradation products of degradable, synthetic scaffolds following macrophage-mediated degradation of such materials [17]. Since these materials degrade upon implantation, macrophages secrete effector molecules such as IL-6, IL-1B, TNFa, TGFB, and IL-10, which bind dendritic cell receptors and can drive differential responses. For example, it has been shown that

macrophage secretion of IL-6 can maintain a dendritic cell in an immature state via STAT3 activation [18]. In contrast, TGFB secretion from macrophages can promote dendritic cell signaling to T cells, promoting a regulatory T cell phenotype [19]. In other words, dendritic cells are a required mediator between the innate and adaptive arms of the immune response, both of which contribute to the clinical outcome when biomaterials are used. Direct modulation of dendritic cell responses is an emerging field in biomaterial and pharmacologic development.

T and B cells

Though the innate immune system is the first responder to any implanted material, the adaptive immune system has been shown to play a critical role in the determination of outcomes and in facilitating proregenerative cross-talk with cells of the innate immune system. Though historically T and B cells have been associated with tissue and organ graft acceptance or rejection, antibody formation, and immunologic memory, these cell types are now recognized to play a role in resolving inflammation and promoting wound healing. Th1 and Th2 type T cells have been shown to propagate M1- and M2-like macrophage phenotypes through the secretion of cytokine networks. T cells also play a role in activating other cells of the immune response, particularly eosinophils [20]. Particular biomaterials and their degradation products have specific effects upon T and B cells. For example, polymers with cationic properties have been shown to activate a Th1 type adaptive immune response [21]. Regulatory T cells (T_{regs}) have been shown to play a role in skin wound healing [22] and myocardial infarction [23] by promoting a tolerant immune cell microenvironment and activating proremodeling phenotypes in other effector T cells and macrophages. T and B cells can secrete cytokines that in some cases promote tissue remodeling and in others propagate fibrosis or tumorigenesis [24]. These disparate phenotypes highlight the importance in identifying the signaling molecules, including cytokines, and their associated mechanisms rather than categorizing immune response types simply as type 1 or type 2, since such cytokines can cause paradoxical effects in different microenvironments.

Specialized immune cell functions beyond host defense

Beyond their ubiquitous role in responding to a foreign body, such as a biomaterial or a tissue engineered construct, immune cells play specialized roles in nearly all body systems. For example, alternatively activated macrophages have been shown to contribute to thermogenesis in adipose tissue via IL-4 signaling [25]. Macrophages contribute to iron recycling and delivery to hepatocytes in the liver [26]. Brain resident macrophages, microglia, are required to maintain synaptic health and contribute to normal neuronal development [27]. Macrophages also regulate cardiac conduction in the AV node [28]. Immune cells, such as macrophages, eosinophils, and T cells, can create coordinated cytokine networks that are powerful determinants of tissue remodeling after injury. For example, prostaglandin E2 secretion into the stem cell niche is required for efficient myogenesis via myoblasts and satellite cells [29]. Cytokines that are categorized as alarmins, such as IL-33, play diverse roles in tissue homeostasis, disease progression, fibrosis resolution, and allergic reactions [30]. Many alarmins are considered double agents, as their signaling mechanisms can promote both proinflammatory and proremodeling effects dependent on their localization and the signaling pathways they activate [31].

Recent work has revealed that immune cell signaling both with other immune cells and with stem/progenitor cell populations goes beyond cytokine networks and proteolytic enzyme release and includes the release of a wide variety of extracellular vesicles (EV). Much of the research surrounding the release of EV focuses on stem-cell EV release. However, not only do stem-cell associated EV send potent signals to immune cells but also immune cells may release specialized EV of their own. EV are known to contain potent bioactive signaling molecules, including microRNA, proteins, lipids, and nucleic acids [32]. The role that EV plays in disease pathogenesis, tissue homeostasis, and potentially disease resolution is becoming more clear, and a better understanding of how EV interact with immune cells could provide new insights to tailoring TE and RM therapies. For example, tumor-associated macrophages have been shown to release the EV that are enriched with certain microRNA that potentiate breast cancer cell invasion [33]. Conversely, delivery of EV to macrophages can selectively prompt their release of proinflammatory or proremodeling cytokines [34]. In fact, the renewed interest in studying EV and their signaling mechanisms, particularly exosomes, stems from important findings showing exosome release from antigen-presenting cells, B-lymphocytes, in particular, which can induce MHC-class II specific responses [35]. These findings, among other recent findings, suggest that natural processes, such as the secretion of EV, that can be manipulated for promoting constructive and functional tissue remodeling outcomes.

Tissue engineering/regenerative medicine strategies as immunotherapy

The increasing complexity of immune cell function and interactions with other cell types has shifted the

perspective with which tissue engineers evaluate and analyze the immune response to their constructs. Instead of focusing on circumvention of an FBR through the development of "inert" biomaterials, a term that is arguably self-contradictory, TE strategies can now be considered as immunotherapies, specifically designed to manipulate immune cell function.

The FBR has been considered a limiting factor to implanted materials [36]. It is now well established that biologic materials derived from mammalian ECM, when properly prepared, can mitigate the FBR and promote improved tissue remodeling outcomes. In contrast, synthetic biomaterials are typically associated with a robust FBR [37]. Research over the past decade has uncovered clues as to why such divergent results occur. As previously mentioned, biomaterials derived from ECM promote a favorable macrophage response at early time points that is predictive of downstream constructive remodeling, but the specific component(s) responsible for these effects is unknown. The answer is slowly emerging through the understanding of how external factors, including material processing and patient-related factors, can affect the host response. For example, cross-linking ECM bioscaffolds negatively affects the macrophage response [38]. Though this less favorable response could be a direct result of the chemicals used for cross-linking, studies have shown that a lack of scaffold degradation and associated absence of release of bioactive degradation products may be the real culprit [39,40]. Other factors, such as source tissue [41], source animal age [42], and terminal sterilization [43], have also been shown to play a role in dictating both the macrophage response and downstream outcomes to ECM-based materials. External factors, such as incorporation of site-appropriate mechanical loading at the biomaterial implant site [44], can markedly change the macrophage and remodeling response. Taken together, tissue engineers can tailor the design and processing methods for superior outcomes as a first step to positioning biomaterials as immunotherapies. The choice of scaffold material utilized can also have profound effects upon the immune response.

One example of biomaterials designed to modify the immune response involves cytokine signaling to promote better host tissue integration. IL-4-coated polypropylene mesh materials can shift the early macrophage response following implantation toward an M2-like phenotype and diminish downstream fibrosis [45]. The use of polyethylene glycol hydrogels to deliver cytokines, such as TGF-B or IL-10, has been developed to modulate dendritic cell activation states [46]. Other groups have evaluated combinatorial approaches delivering more than one small molecule, cytokine, or pharmacologic agents to promote tissue remodeling processes such as angiogenesis [47]. Such approaches highlight the feasibility and proof-of-concept in modulating the immune response to promote better outcomes.

Future considerations for immune cell targeting tissue engineering/regenerative medicine therapies

Targeting immune cells and their associated signaling is an attractive and potentially powerful approach for the field of TE. There is now convincing evidence of the constructive cross-talk that exists between stem cells and the immune system. However, effective methods of utilizing this phenomenon to promote functional tissue formation have yet to be identified. However, as the understanding of how immune cells work in concert with tissue building efforts, and as the toolkit with which to identify, manipulate, and evaluate those roles expands, TE technologies are primed for clinical translation to treat a variety of disease states.

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Chapter 21

Tissue engineering and transplantation in the fetus

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Introduction

The first successful interventional procedure performed on a fetal patient took place in 1963, when Sir William Liley transfused red blood cells into the peritoneal cavity of a fetus suffering from erythroblastosis fetalis [1–3]. It was not until nearly 20 years later, in 1982, that Dr. Michael Harrison and his colleagues at the University of California, San Francisco (UCSF) performed the first successful in utero surgical procedure [4] to correct a patient with congenital hydronephrosis. It was this landmark achievement that ushered in a whole new era of prenatal medicine. Since that time, tremendous progress has been made in both open fetal surgery procedures and in fetoscopic intervention, due in large part to Dr. Harrison's remarkable drive and spotless integrity, which led him to establish an international fetal-treatment registry, in which he urged physicians to report all outcomes of any attempted fetal procedures, with the goal of establishing "the benefits and liabilities of fetal therapy as soon as possible" [5]. As a direct result of these efforts, physicians are now able to treat/correct a variety of anatomic anomalies that would previously have been fatal or sentenced the unborn child to a lifetime of disability [6,7].

To provide a few examples, a great deal of progress has made in the in utero surgical correction of lower urinary tract obstruction, which is a common congenital abnormality occurring in up to 1% all fetuses [8–16], surgical correction of congenital diaphragmatic hernia [3,16–23], surgical debulking of very dangerous and often prenatally fatal sacrococcygeal teratoma [3,16,18,23–28], both "open" and fetoscopic-based approaches to correct congenital cystic adenomatoid malformation and congenital pulmonary airway malformations [24,29–32], and even efforts to surgically correct neural tube defects such as myelomeningocele (MMC/spina bifida aperta) [33–40]. Looking specifically at MMC, recent exciting results in animal models have highlighted the potential of novel tissue engineering-based approaches using injectable scaffolds, either alone or seeded with stem cells to create so-called stem-cell patches [41–46]. These new noninvasive approaches to treating MMC are particularly appealing, because they could achieve the watertight tissue closure that is needed to prevent damage to the spinal cord much earlier in gestation than the current open surgery-based methods can be performed. As such, the potential benefit to the patient is greatly enhanced [16].

Despite the remarkable differences, such advances have made for these patients and their families, it is important to note that, even when using approaches such as fetoscopy that are considered to be relatively noninvasive, these surgery-based methods still carry a high risk of premature rupture of membranes (PROM)/preterm prelabor rupture of membranes (PPROM), with resultant preterm birth and fetal demise [16,18,23]. A joint endeavor undertaken by investigators at UCSF, UC Berkeley, and Caltech recently led to the development of a highly promising biocompatible adhesive called Amnioseal, which is injected prior to performing fetal surgical procedures to preseal the amniotic membranes and thereby prevent PROM/PPROM [47], which would be a true game-changer in the field of fetal surgery.

Importantly, however, even if this new biomaterial proves completely effective at preventing PROM/PPROM, fetal treatments that are based on surgical intervention will still be intrinsically limited to the treatment of disorders involving/caused by structural abnormalities [16,23,48,49]. In contrast, in utero transplantation (IUTx) and in utero gene therapy (IUGT) offer the possibility of

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treating, and ideally curing, a wide range of genetic disorders. With the advent of high-resolution ultrasonography and exquisitely sensitive, high-throughput molecular techniques, the vast majority of congenital conditions can now be diagnosed early in gestation using fetal cells or even trace amounts of cell-free fetal DNA that are present in the maternal blood [50]. As such, diagnoses can now be made with vanishingly small risks to the fetus and the mother.

Of note, these remarkable advances in prenatal imaging, molecular diagnostics, and fetal surgical techniques have not only improved the ability to identify diseases early in development, they have also made it possible to safely deliver stem cells and/or gene therapy vectors to precise anatomic sites within the early-gestation fetus. Preemptive treatment of the fetus by IUTx or IUGT would completely transform the paradigm for treating genetic disorders [6], allowing physicians to intervene prior to clinical manifestations of disease, an approach that could promise the birth of a healthy infant who required no further treatment. In addition to the obvious psychological benefits of curing a disease in utero, the elimination of the need for lifelong, noncurative treatment would have a profound impact on the quality of life (QoL) of the patient, and his/her family, as well as dramatically reducing the cost burden for society. It is critical to note that there are also several biological advantages unique to fetal development, which provide compelling reasons to believe that stem-cell transplantation and/or gene therapy would be far more efficient and effective if administered during fetal life rather than postnatally.

In this chapter, we present the therapeutic rationale for the use of IUTx and IUGT, and we review key experimental evidence to support their use. We discuss some of the unforeseen biological barriers that have thus far precluded more widespread clinical application/success of IUTx, and we provide an overview of IUGT, illustrating some of the unique advantages it offers as a treatment modality, as well as some of the potential risks to be addressed prior to clinical implementation. We showcase the hemophilias as a prime example of a genetic disease that could be corrected via IUTx and/or IUGT, and we finish the chapter by highlighting several recent breakthroughs that promise to move these exciting therapeutic approaches into the clinic in the near future.

Rationale for in utero therapies

Long before scientists even considered performing in utero treatments, a naturally occurring phenomenon established critical proof-of-concept for the potential of IUTx by providing irrefutable evidence that introducing foreign cells early during fetal development could lead to lifelong engraftment and induce durable immune tolerance to the cellular donor. This remarkable discovery was made in 1945 by Owen [51], who perceptively observed that the shared placental circulation present in monochorionic dizygotic cattle enabled intrauterine exchange of circulating hematopoietic stem cells (HSC) and that this produced life-long hematopoietic chimerism, which resulted in donor-specific tolerance to the sibling twin [51–53]. This extraordinary finding in cattle was of tremendous interest from a biological standpoint. However, it was the subsequent observation that natural chimerism also occurs in both nonhuman primate (NHP) and human twins [54–59] and that this chimerism, just as Owen observed in cattle, led to a lack of alloreactivity between the two siblings [54,59], which established the clinical relevance of this intriguing natural phenomenon. Even more striking was the finding that, in the case of dizygotic human twins, the levels of chimerism that have been observed have often been high enough that they would be predicted to exert a therapeutic effect in most hematologic diseases [60]. Collectively, these exciting findings in nature have long provided the scientific basis for the promised therapeutic potential of IUTx, and for its ability to induce donor-specific immune tolerance and have fueled efforts to bring this therapy to clinical fruition.

While these "experiments of nature" provided proof-of-concept for IUTx, why would such an approach be clinically useful or offer benefits over existing postnatal approaches to therapy? To answer these questions, we must first consider the fact that many genetic diseases exert a significant amount of irreversible damage during embryonic and fetal development. For example, substantial neuronal damage is associated with inherited metabolic diseases such as Gaucher's, Lesch–Nyhan, and Tay Sachs. In these patients, even state-of-the-art treatment given postnatally only mediates a limited therapeutic benefit, since it cannot reverse the damage that the disease has exerted during development. By far, the most compelling rationale for IUTx (and IUGT, as we discuss later) is the ability to treat these diseases early enough in development to prevent disease onset and thereby evade the devastating manifestations that would otherwise occur before birth. It is important to note that, even in patients with diseases that can be treated postnatally, compelling psychological and financial benefits exist to correct such diseases in utero, since prenatal intervention would allow the birth of a healthy infant, who, ideally, would require no further treatments.

There are also several indisputable physiological/biological reasons to believe that treating in utero would be easier and produce a more pronounced effect than waiting until after birth to intervene. It has long been appreciated that normal developmental events that occur within the nascent hematopoietic/immune system create several unique opportunities that may facilitate the engraftment

of foreign cells and eliminate the need for the myeloablative conditioning required with postnatal hematopoietic transplantation, thereby avoiding the complications and significant risks inherent to this toxic procedure [61–64] (reviewed in [60]). We will, therefore, begin our dialogue about in utero therapies by providing a brief history of the origins of IUTx, highlighting some of the key experimental advances that have been made in recent years, and summarizing the clinical IUTx studies that have been performed.

In utero transplantation

Although several cell types have been considered and explored in the context of IUTx, this chapter will focus predominantly on HSC, which represent the prototype stem cell within the body. HSC are multipotent stem cells that undergo self-renewal and multilineage differentiation to generate all of the mature hematopoietic lineages and thereby maintain functional hematopoiesis throughout fetal and adult life [65,66]. As such, they are well suited for treating a broad range of hematopoietic disorders, and the successful transplantation of HSC can result in lifelong disease correction. Since their discovery, HSC have been the focus of intensive research and have proven clinically invaluable to restore hematopoiesis following inadvertent radiation exposure and following radio/chemotherapy to eliminate hematologic tumors. HSC are the most extensively characterized stem cells in the body, and much of what we know about the biology and behavior of stem cells in general is based upon the paradigm established with studies on HSC. In light of their remarkable properties, it is not surprising that HSC were the first cell type tested in IUTx, and they are the cells that have been used in the vast majority of both experimental and clinical IUTx studies to-date.

Postnatal HSC transplantation is a lifesaving procedure that can cure numerous malignant and benign hematologic disorders. Unfortunately, however, its execution is not without problems and significant risks to the patient, as a suitable, immunologically compatible donor must be found (which is often not possible), and the pretransplant conditioning regimen and the posttransplant period of leukopenia, during which the patient is severely immunocompromised, often result is serious morbidities that can prove fatal, even in the hands of an experienced transplant team. The developing fetus provides a unique opportunity to overcome these issues, due to the large-scale migration of stem cells that occurs to seed tissue compartments during ontogeny. Definitive hematopoiesis commences in the yolk sac and/or aorta–gonad–mesonephros region, migrates to the fetal liver, and finally shifts to the bone marrow (BM), where it then resides for the remainder of life [67–69]. Much of the initial rationale for attempting HSC transplantation in utero was based on the hope that it might be possible to "piggyback" on these naturally occurring migrations and exploit the nascent hematopoietic niches arising during development to achieve efficient engraftment, differentiation, and expansion of donor HSC without the need for myeloablation. If this supposition proved correct, IUTx would enable donor reconstitution of the defective hematopoietic compartment and correction of the disease while eliminating one of the primary causes of the marked morbidity and mortality associated with postnatal HSC transplantation.

Unfortunately, experiments over the last 20 years have made it clear that this expectation was naively optimistic. Once it became apparent that the endogenous circulating fetal HSC are present in relatively large numbers and that they possess higher proliferative and repopulating capacity than their adult (donor) counterparts [70–72], scientists came to appreciate that the fetal hematopoietic system is, in fact, highly competitive, and therefore poses a formidable obstacle to engraftment of transplanted adult HSC. However, it was still hoped that, with better understanding, it might one day be possible to manipulate the regulatory signals controlling the migrations of HSC and their seeding of nascent marrow niches to tip the scale in favor of the donor cells [73].

Looking beyond the unique biological characteristics of the fetal recipient, the very small size of the fetus also offers a distinct logistical/technical advantage compared to an adult or even a pediatric patient in the context of an HSC transplantation. IUTx would ideally take place at about 12 weeks of gestation, at which point the human fetus weighs roughly 35 g [6,7,50,60,74,75]. By virtue of its extremely small size/mass, a much larger cell dose can be transplanted on a per kilogram basis than could ever be achieved after birth. Another major advantage of the fetal environment is the isolation of the fetus within the sterile environment that exists within the uterus. This is particularly important if one considers treating an immunodeficiency, since performing the transplant in utero would enable the maternal womb to serve as a sterile isolette, sheltering the fetus from pathogens while its immune system is reconstituted from the transplanted HSC [6].

As discussed in the preceding section, Owen's experiments of nature highlighted another aspect of fetal biology that provides what is perhaps the most compelling reason to perform transplantation or gene therapy in utero: the ability to induce immune tolerance to the cell donor or the vector-encoded transgene product(s) [61]. Early in gestation, the nascent immune system undergoes a course of self-education that occurs primarily in the fetal thymus. This process consists of both a positive selection of prelymphocytes that recognize "self"-major histocompatibility complex (MHC) and the deletion of any prelymphocytes that recognize, with high-affinity, any of the innumerable

fetal self-antigens in association with self-MHC [76,77]. Ideally, this process creates an immune system that is devoid of self-reactive lymphocytes and yet retains the diversity required to recognize any foreign antigens in association with self-MHC [60,77]. It stands to reason that, if allogeneic cells were introduced during this time via IUTx, donor antigens should be presented in the thymus along with the host's own self-antigens as part of this naturally occurring education process, leading to the deletion of any alloreactive T-cells, and thereby creating donor-specific immune tolerance. The induction of tolerance following IUTx is important for two reasons. First, the presence of tolerance should facilitate the maintenance of the donor hematopoietic cells. Perhaps even more importantly, a growing body of data supports the exciting possibility that, even if the levels of donor cell engraftment following IUTx are not sufficient to be curative/therapeutic, the antigen-specific tolerance induced by IUTx may enable the administration of postnatal "booster" transplants of same-donor HSC to achieve therapeutic levels of engraftment without the need for toxic myeloablation. It has also been suggested that this state of immune tolerance to the HSC donor might even make it possible to later perform transplants of same-donor solid organs (should it prove necessary) without fear of rejection [78–83].

Early murine experiments with in utero transplantation

Billingham et al. [61] performed groundbreaking studies in mice in the 1950s that provided the first experimental evidence that IUTx of allogeneic cells induced donor-specific tolerance that was robust enough to enable successful postnatal skin grafting. After this pivotal demonstration, work over the next two decades was largely focused on naturally occurring chimeras and better defining the potential for in utero chimerism to produce immune tolerance, and the fledgling field of IUTx would have to wait until the end of the 1970s before Fleischman and Mintz would publish the first report of successful hematopoietic chimerism following IUTx, and provide the first exciting experimental proof that IUTx could reverse a genetic disorder. To accomplish this remarkable achievement, Fleischman and Mintz used a line of mice with genetic anemia caused by c-Kit deficiency to make three important observations: (1) transplanting adult allogeneic BM stem cells into the placenta of these mice at E11 reversed the genetic anemia; (2) the degree of erythroid replacement correlated with the degree of underlying anemia; and (3) erythroid reconstitution could remarkably be achieved in this model following the transplantation of just a single HSC [84,85]. It was these early studies asking questions about basic stem-cell biology that identified host cell competition as a barrier to donor cell engraftment after IUTx. As we will discuss in detail shortly, this unexpected phenomenon was to prove critical to the clinical implementation of IUTx.

Almost another two decades would pass before Blazar's team [86] would confirm the ability of IUTx to achieve multilineage chimerism in stem cell–deficient recipients. However, in contrast to the erythroid-specific defect studied by Fleischman and Mitz, Blazar's group demonstrated that when mice with a T-cell proliferation and survival defect due to severe combined immunodeficiency (SCID) were treated by IUTx, the engraftment of the transplanted donor cells was restricted to the lymphoid lineage, a phenomenon termed "split chimerism" [87,88]. These studies thus provided the first evidence that host cell competition could limit donor cell engraftment following IUTx, and further solidified the concept that, when a lineage-specific deficiency is present, IUTx can yield nearly complete reconstitution of the defective lineage yet contribute only negligibly to the other hematopoietic lineages. These findings thus hinted that achieving clinically meaningful levels of donor cell engraftment after IUTx in recipients with a competitive hematopoietic compartment was likely going to be far more difficult that had been assumed. This notion was strengthened by subsequent studies showing that IUTx in immunocompetent, wild-type mice often led to very low engraftment rates, while studies in immunodeficient recipients uniformly yielded far more encouraging results [84,86,87,89].

In utero transplantation experiments in large preclinical animal models

Since these initial trailblazing murine studies were performed over three decades ago, a great deal of progress has been made in the field, not only in mice, but in larger preclinical/ translational animal models as well. Sheep was the first large animal model in which successful engraftment of allogeneic cells was demonstrated following IUTx [90]. Of the available animal models, sheep have been a particularly valuable preclinical tool for the study of IUTx. Fetal sheep provide a natural, unperturbed environment in which to study IUTx. Moreover, since even xenogeneic cells are not rejected if IUTx is performed early enough in gestation, it is possible to study the engraftment and differentiation capacity of a variety of human stem cells in this system [91–100]. Sheep share many important physiological and developmental characteristics with humans. As a result, they have been used extensively in the study of mammalian fetal physiology, and the results obtained with this model have been directly applicable to the understanding of human fetal growth and development [101].

Like humans, sheep typically give birth to only one or two offspring in each pregnancy. This is in marked contrast to dogs, cats, pigs, and many other large animals that have large litters of offspring. A major complication that arises with translating approaches that prove successful in mice and even more modestly sized animal models to humans is the issue of accurate scale-up of cell/vector dosage per kg body weight. A typical human patient is roughly 2500 times larger than a mouse [102], making linear scale-up of dosage based on body-weight challenging, if not impossible. Importantly, sheep are similar in size/weight to humans, both at birth (7−9 lb) and as adults (150−200 lb), making it possible to develop and test clinically relevant doses of cells/gene therapy vectors directly in this model prior to translating to the clinic. In addition, the development of the ovine hematopoietic and immune systems during fetal ontogeny has been delineated in great detail, and it has proven to be quite similar to that of humans [103−109], making this model ideal for investigating the immune facets of IUTx (and IUGT). The long lifespan of sheep (>10 years in captivity) allows the important issues of long-term efficacy and safety to be addressed with far greater rigor than could ever be accomplished in short-lived animals such as mice.

The fetal sheep model has also played a critical role in defining the phenotype of long-term engrafting human HSC, enabling the identification of several novel human stem-cell markers/phenotypes (e.g., CD34 + CD38 −, HLA-DR −, Thy-1 +, CD133, KDR, and CD34 −) [110−119], many of which are now in clinical use, attesting to the high translational value of data obtained with this preclinical model. This system has also proven invaluable for defining the role the various hematopoietic niches within the recipient marrow play in the engraftment of HSC following IUTx [101,120−122], and it has proven itself as a preclinical model for studying the process of HSC mobilization, since engrafted human hematopoietic cells respond to mobilizing human cytokines in a manner that is similar to their native counterparts [100,123−126]. These collective data provide strong support for the value of the fetal sheep model for developing/testing IUTx/IUGT approaches and obtaining results of high clinical relevance [75,127,128].

Like sheep, dogs are also receptive to fetal transplants of xenogeneic cells, and low-level multilineage hematopoietic engraftment has been achieved following IUTx in hematologically normal dogs [80]. Flake's group used the canine model to provide compelling evidence of the therapeutic potential of IUTx by showing that the levels of hematopoietic engraftment following IUTx are sufficient to (1) correct the clinical phenotype of the canine analog of human leukocyte adhesion deficiency; and (2) induce donor-specific tolerance (in some animals) that permitted postnatal "boosting" of chimerism using a mild conditioning regimen, followed by transplantation of same donor T cell−depleted marrow [64,80,129,130]. In addition to studies in mice, sheep, and dogs, IUTx has also been successfully performed in goats [131,132], pigs [80], and NHPs, although the levels of engraftment achieved in the NHP model were relatively low [132−138]. Subsequent studies in the porcine model provided compelling evidence that inducing immune tolerance in the fetus is highly beneficial for postnatal solid organ transplantation, as IUTx of adult BM−derived HSC in fetal pigs prolonged the survival of a kidney allograft [139]. These studies thus provide important experimental support for the possibility of using this strategy in fetuses with congenital abnormalities that require postnatal organ transplantation.

Despite the undeniable advantages of performing translational research in large animal models that more closely mimic human patients, there are important questions that are just not feasible to attempt to answer in large outbred animals. Due to the ease and exquisite precision with which they can be genetically manipulated, murine models have been used to interrogate various aspects of the immune system to gain an understanding of the mechanism(s) of tolerance induction following IUTx. Data from Flake, MacKenzie, Peranteau, Shaaban, Nijagal, et al. published over the last decade have collectively provided compelling evidence that clonal deletion, anergy, and induction of donor-specific Tregs all appear to play critical roles in the establishment of chimerism and the induction of immune tolerance [79,81,83,140−145]. Interestingly, these studies in mice have demonstrated that stable engraftment of even low levels of allogeneic HSC (1%−2% engraftment) can lead to postnatal tolerance across full MHC barriers, and have revealed that tolerance induction depends upon achieving a threshold level of engraftment and on maintaining chimerism in the host [146]. Elegant recent work from Shaaban et al. has indicated that Natural Killer (NK) cell tolerance appears to play a key part in establishing this engraftment threshold [147−150].

While similar mechanistic studies are clearly needed in preclinical large animal models, as the human immune system may well present its own set of unique challenges, these collective results demonstrate the technical feasibility of IUTx, confirm its ability to induce donor-specific immune tolerance, and shed light on some of the requisite pathways to tolerance induction, establishing an essential foundation for ultimate clinical application of IUTx.

Barriers to in utero transplantation success

An improving understanding of the ontogeny of the fetal immune system combined with the evidence provided by the preceding studies led to the seemingly sound premise

that a so-called "window of opportunity" [62] should exist, prior to the appearance of mature T-cells in the fetal thymus and circulation (~12–14 weeks of gestation in the human fetus) [77], during which the fetus should be "preimmune" and receptive to allogeneic (or even xenogeneic) cells/tissues. However, as the interest in IUTx grew, experimental evidence began accumulating that made it clear that significant barriers existed that prevented successful engraftment after IUTx if the recipient did not possess a lineage-specific defect, such anemia or SCID, that bestowed a competitive advantage to the donor cells [62,143,151–154]. In 2007 Peranteau et al. [155] performed critical studies that toppled the long-standing belief that the fetus is truly "preimmune." In this report, it was shown that when cells from congenic donors were used for IUTx, mice could more consistently be engrafted, and at higher levels, compared to when IUTx was performed with phenotypically identical cells from allogeneic donors. Moreover, when allogeneic cells were used, only ~30% of the recipients consistently exhibited chimerism. These surprising findings thus suggested that the fetal immune system represents one of the major barriers that limit HSC engraftment following IUTx. The authors went on to perform elegant tracking studies that demonstrated that although 100% of allogeneic and congenic recipients maintained high levels of engraftment up to 3 weeks after IUTx, between 3 and 5 weeks post-IUTx, 70% of allogeneic animals lost their engraftment, while 100% of congenic animals remained chimeric [155].

The authors then demonstrated that both cellular and humoral adaptive alloresponses were present in nonchimeric animals, which led to the conclusion that the host (fetal) immune response was responsible for limiting donor cell engraftment. This report was troubling, as it was in seeming contradiction with a wealth of prior data, from multiple groups, including these authors, which had demonstrated that IUTx, in both mice and other animal models, led to successful long-term chimerism in a percentage of recipients and often led to deletional tolerance to donor antigens. A possible explanation for these differing findings came from the intriguing observation that if pups that received IUTx were placed with surrogate mothers that had not been exposed to donor alloantigens, all recipients maintained their chimerism [140,156]. These findings thus provided evidence that an immune barrier to IUTx existed not only as a result of the nascent fetal immune system but also that of the mother and suggested that IUTx may have the potential to trigger maternal alloimmunization. The authors went on to theorize that this maternal alloimmunization led to the subsequent transfer of allo-antibodies (Abs) to the pup via breast milk, which induced an adaptive alloresponse in the pup with a subsequent loss of chimerism. Perhaps most importantly, this study confirmed that in the absence of a maternal immune response, either via foster nursing or through the use of maternal donor cells, engraftment and tolerance were uniformly present via a mechanism of partial deletion of donor-reactive T-cells and the induction of a potent T-regulatory cell response.

An independent series of murine IUTx studies, performed at around this same time by Nijagal, MacKenzie, et al., corroborated the finding of maternal alloimmunization as a result of the fetal intervention but found that subsequent maternal–fetal T-cell trafficking was the main factor responsible for the loss of chimerism [141,142,157–159]. Obviously, placentation, maternal–fetal trafficking of Abs and cells, and the time course of events after IUTx are considerably different in mice when compared to large animal models or during human pregnancy. Nonetheless, these findings raise the important question of whether maternal immunization is an issue in large animal models and clinical circumstances, and whether it is a limitation to hematopoietic engraftment following IUTx. Until this question is addressed, these findings have led to the informal consensus in the field that it may be prudent to use maternal cells in any clinical application of IUTx to remove the possibility of triggering a maternal immune response.

While the data from these studies are certainly compelling, and the methods used were unassailable, Shaaban et al. astutely noted that the maternal immune system has been intact in all human patients who have thus far undergone IUTx for a variety of diseases, despite the nature of the clinical outcome (success or failure); data which they feel supports the conclusion that the maternal immune response cannot be a key determinant in IUTx-related engraftment failure [147,148]. In an effort to resolve/explain this incongruity between mouse data and clinical outcomes, this group has spent many years investigating the ontogeny of the fetal immune system to define the immune cells and pathways present at the time of IUTx that could serve as a barrier to engraftment of allogeneic cells. These authors have identified a subset of early NK cells within the fetal liver that express adult levels of alloreactive receptors, suggesting that NK cells may pose a barrier to engraftment of transplanted cells as early as the end of the first trimester in humans. Indeed, depleting NK cells from the fetus, but not from the mother, enabled reliable engraftment of allogeneic cells following IUTx [147,148,150]. Interestingly, the levels of early chimerism required to induce NK cell tolerance (1%–2%) agree exactly with the threshold levels discussed earlier, likely due to a mechanistic link that exists between the induction of prenatal NK cell tolerance and the process of trogocytosis [149]. While these important findings will have to be reproduced in large animal models of IUTx, these elegant and highly mechanistic studies collectively provide a very persuasive argument for the importance of fetal NK cells in the ability to achieve engraftment of

allogeneic cells following IUTx, just as has been seen in postnatal HSC transplantation [160].

To further complicate the already perplexing issue of the role of the fetal and maternal immune systems in acceptance of hematopoietic allografts following IUTx, recent work from the Peranteau lab has now added to the compelling evidence for the importance of the maternal immune system in the ultimate fate of hematopoietic cells following IUTx. In these studies, the authors showed that the presence of preexisting maternal Abs to antigens present on the transplanted cells leads to very rapid (within 4 hours) prenatal rejection of allotransplants following IUTx in a murine model system [161].

In addition to the fetal and/or maternal immune response, perhaps the most important perceived barrier to engraftment of allogeneic HSC is host cell competition. In the setting of postnatal HSC transplantation, the recipient receives myeloablative conditioning prior to donor cell infusion, to suppress endogenous hematopoiesis and, perhaps, free spaces within the hematopoietic niches of the BM. In marked contrast, following IUTx, the donor cells must compete against the robust fetal hematopoietic compartment. The idea that donor (adult) cells may have a competitive disadvantage in the fetal environment is supported by the ease with which high levels of donor hematopoiesis can be achieved in c-Kit-deficient mice, in which as few as one or two normal HSC can fully reconstitute the hematopoietic compartment after IUTx [84]. Studies of IUTx performed in SCID mice also illustrate the importance of host cell competition [87,162]. In this model, in which donor lymphoid cells have a survival and proliferative advantage, IUTx results in complete reconstitution of the lymphoid compartment with minimal engraftment of other unaffected lineages.

These preceding experimental data illustrate just how effective this competitive advantage can be in the setting of a proliferative defect in one or more lineages. However, when no defect in host hematopoietic vigor is present, the scale tips in favor of the endogenous fetal HSC, which have a marked competitive advantage over their adult-derived counterparts [70,163–166], due to their accelerated/enhanced cycling and expansion kinetics. Data from the congenic mouse model of IUTx provide a striking example of the degree to which this competition limits long-term donor cell engraftment. Even in this setting, in which no immune barriers exist, long-term donor cell engraftment levels remained below 10% following the delivery of mega-doses of donor cells (2×10^{11} donor cells/kg) [155].

The limited number of available niches and the proliferative capacity of the fetal environment have also been implicated as a barrier to success with IUTx [82]. Favorable competition of transplanted HSC with the host cells for available hematopoietic niches is essential for successful engraftment, as evidenced by the enhanced success of IUTx when more competitive fetal donor cells or larger doses of adult cells are used [73,167]. Improved competition for available host niches would obviously be predicted to lead to higher levels of early chimerism, as is seen in adult mice, in which selective depletion of host HSC before BM transplant results in high rates of engraftment [168]. However, no direct evidence exists to support the existence of quantitative or qualitative differences in the number of HSC or available niches between recipients with SCID and those with sickle cell disease or β-thalassemia (reviewed in Refs. [169,170]), or any of the range of other disorders that have proven refractory to correction by IUTx. As such, it is hard to envision how a competitive niche model could explain the conflicting observations for immunodeficient versus nonimmunodeficient recipients, with respect to the success of donor cell engraftment following IUTx [148]. Nevertheless, the possibility that there are a finite number of available hematopoietic niches for donor cell engraftment following IUTx is supported by the finding that increasing the dose of donor cells results in an eventual plateau of engraftment efficiency in an allogeneic and xenogeneic fetal sheep model [171].

Using sheep as a large animal model of IUTx, we demonstrated that administering allogeneic adult BM–derived mesenchymal (CD146 + CXCL12 + VEGFR2 −) or endothelial (CD146 + CXCL12 + VEGFR2 +) cells prior to, or in combination with, HSC, resulted in robust CXCL12 production within the fetal marrow environment, and significantly increased the levels of hematopoietic engraftment. Moreover, the increased expression of VEGFR2 in the microvasculature of CD146 + CXCL12 + VEGFR2 + transplanted animals enhanced the levels of donor-derived hematopoietic cells in circulation. These studies provide important insights into IUTx biology and demonstrate the feasibility of modulating/enhancing the resident marrow niches to enhance HSC engraftment to levels that would likely be therapeutic in many candidate diseases for IUTx [172].

In other studies, maternal administration of busulfan 6 days prior to IUTx was shown to significantly increase engraftment in fetal sheep [173]. While informative, it is not clear whether the toxicities associated with the use of a myeloablative agent during pregnancy would be clinically acceptable. However, recent studies from MacKenzie et al. demonstrated that selective in utero depletion of host HSC using an Ab against the c-Kit receptor (ACK2) results in therapeutic levels of engraftment after neonatal transplantation [174], without any of the cytotoxic effects of an agent like busulfan.

Collectively, these studies support the notion that augmenting host stem-cell niches or inducing resident HSC to vacate the existing niches may both represent viable means of improving chimerism after IUTx. Clearly,

however, further studies are needed to better understand this important issue and develop methods of optimizing the benefits on donor HSC engraftment while ensuring the safety of the fetus and mother.

Clinical experience with in utero transplantation

The early experimental success of IUTx in various animal models generated a great deal of excitement and led to many clinical attempts around the world to treat various hematologic disorders with IUTx. In humans, the first successful IUTx was performed for bare lymphocyte syndrome [175]. Following this seminal case, successful transplantation of fetuses with SCID was also achieved in a number of centers [176–179]. In these cases, fetal liver, paternal BM, or maternal BM–derived CD34 + cells were transplanted between 16 and 26 weeks of gestation and resulted in engraftment of donor cells at birth, and clinical improvement. To date, IUTx has been performed on 48 human patients for 14 different genetic disorders, including hemoglobinopathies, chronic granulomatous disease (CGD), Chediak–Higashi syndrome, and inborn errors of metabolism [62,75,180–183] (reviewed in Ref. [50]). These studies have collectively provided irrefutable proof that the early human fetus can safely be accessed multiple times, assuming that a minimally invasive, ultrasound-guided approach is employed [62,75,156,176,180,181,184].

Unfortunately, with the notable exception of patients with SCID, the clinical experience thus far with IUTx has been largely disappointing. However, SCID is a unique disorder that provides a survival and proliferative advantage for donor T-cells, and the engraftment achieved in these patients has only been documented to reconstitute the T-cell lineage (split chimerism) [75], just as Blazar et al. observed in their early experimental work in mice [87,162]. The results of the 48 clinical IUTx cases performed to-date have clearly demonstrated that IUTx, using currently employed methods, is unable to establish therapeutic levels of donor cell engraftment in recipients whose hematopoietic system exhibits a normal level of competitiveness. Regrettably, the large number of variables and inherent inconsistencies among these reported clinical cases have made it impossible to identify a common factor(s) responsible for the observed poor engraftment and lack of clinical success. As a result, investigators have been forced to perform the previously detailed, rigorous experiments in animal models to gain insight into the barriers that limit engraftment after IUTx.

Since the majority of the anticipated target disorders to be treated via IUTx, such as the hemoglobinopathies and the lysosomal storage diseases, are competitively normal in fetal life, methods must be developed in clinically relevant animal models to overcome host cell competition to improve clinical success with IUTx. A recent study performed in the canine model by Flake's group [64] showed that administering large numbers of highly enriched HSC via an ultrasound-guided intravascular (intracardiac) route resulted in significantly higher levels of engraftment than the intraperitoneal (IP) route that has been used in most clinical studies. Of note, the levels obtained via this new route would be predicted to be therapeutic in most candidate diseases, which has generated a great deal of enthusiasm in the field [185,186]. However, other studies in sheep have produced contradictory results, showing that the intravascular route is no better than the IP route, leading the authors of this other study to conclude that the markedly greater safety afforded by the IP route will likely make this the clinical route of choice [187].

One area that has shown great promise is the notion that the best clinical application for IUTx in the near future may be to use IUTx to induce prenatal tolerance to facilitate nontoxic postnatal BMT [7,60]. This approach significantly lowers the threshold of chimerism required for clinical success, since, as previously discussed, stable engraftment levels of only 1%–2% should reliably induce donor-specific immune tolerance. Unequivocal proof for the therapeutic merit of this approach was recently provided by Peranteau, Flake, et al., who used IUTx to achieve low-level hematopoietic engraftment to induce tolerance, and then performed a postnatal, nonablative "boost" transplantation with same-donor HSC. This tactic yielded sufficient levels of donor cell engraftment to phenotypically correct both β-thalassemia and sickle cell disease in the murine system [188]. These exciting results led the authors to conclude, "if adequate engraftment can be achieved to consistently induce donor-specific tolerance without graft versus host disease (GVHD) in a preclinical model, then clinical trials of IUTx for treating genetic disorders that can be prenatally diagnosed and treated by mixed hematopoietic chimerism, such as the hemoglobinopathies and selected immunodeficiency disorders should be initiated" [188]. This conclusion likely played a key role in the recently commenced clinical trial at UCSF to treat α-thalassemia via IUTx [ClinicalTrials.gov Identifier: NCT02986698].

Rationale for in utero gene therapy

When considering the best way to treat genetic diseases, it is critical to realize that a significant amount of irreversible damage often occurs during embryonic and fetal development. Considering inherited metabolic diseases such as Gaucher's, Lesch–Nyhan, and Tay Sachs as examples, these disorders produce marked irreversible neuronal damage during fetal life, making postnatal treatment of only limited therapeutic benefit, since it cannot reverse the damage that the gene defect exerted during development. Even in patients with diseases that can be

cured postnatally, compelling psychological and financial benefits exist to argue for performing correction in utero, since it would allow the birth of a healthy infant, who, ideally, would require no further treatments. While IUTx can potentially treat many disorders, some genetic diseases may not be amenable to correction by the transplantation of "healthy" stem cells, and for some, it may be preferable to correct the genetic abnormality in situ.

Setting aside the obvious clinical and financial advantages of curing a disease prior to birth, the fetus has many unique properties that make it a far better gene therapy recipient than the adult or even pediatric patient. For example, due to their ability to integrate into the genome of the host cell, γ-retroviruses and lentiviruses have received a great deal of attention as gene delivery vectors, since transduction of a long-lived cell could provide lifelong therapeutic benefit following a single administration. However, one of the main limiting factors to the successful application of these integrating vectors to in vivo gene therapy is the low level of initial transduction and the limited degree of expansion of transduced cells that occurs following gene therapy, since the majority of stem-cell populations in the adult are relatively quiescent and may be inaccessible due to anatomic barriers. During specific periods of development, however, stem and progenitor cell populations are present at relatively high frequencies, and they are accessible to gene transfer, providing a unique window of opportunity to achieve efficient gene transfer to these nascent stem-cell populations, which will be unreachable later in life [7,60]. In the fetus, cells that comprise each of the major tissues/organs are actively cycling to support the continuous growth occurring throughout gestation. Thus most cell types that are largely quiescent in the adult are mitotically active in the fetus, making them amenable to genetic correction with vectors requiring cell division. Furthermore, the active cycling should result in expansion of the gene-corrected cells during the remainder of gestation, allowing even low levels of initial transduction to yield significant levels of gene-corrected cells by birth.

In addition to the ability to access nascent stem-cell populations, just as with IUTx, the immaturity of the fetal immune system should enable delivery of immunogenic transgenes and/or viral vectors that would be rejected by the fully developed postnatal immune system. Many patients suffer from the genetic diseases currently being targeted with postnatal gene therapy because they have never produced a specific protein. As a result, their immune system has never "seen" this protein, and, following postnatal gene therapy, the cells of the immune system identify and destroy any cells in the body expressing the very protein that could cure the patient's disease. Performing IUGT should induce a state of tolerance to the transgene and the viral vector itself, which would not only ensure long-term, stable transduction and expression but should also make it possible to administer postnatal "booster" treatments (if required) with the same vector and transgene without eliciting an immune response.

Furthermore, in similarity to our discussion on IUTx, the extremely small size of the fetus at the proposed time of intervention offers distinct advantages, as it is possible to achieve much higher vector-to-cell ratios than would be possible later in life, which should greatly enhance the efficiency of transduction. In addition, the ability to administer a small volume of vector and achieve the desired rate of transduction is of additional benefit from a technical/logistical standpoint, since the large-scale production of certain vectors under good manufacturing practice (GMP) conditions is often quite challenging.

Hemophilia A as a model genetic disease for correction by in utero gene therapy

Gene transfer using viral vectors exploits the natural ability of the parent virus to efficiently attach to a target cell and transfer its genetic material to the host cell nucleus. To maximize safety, these viral-based vectors are engineered to be devoid of most, if not all, viral genes, which renders the viral vector incapable of replication or expression of potentially immunogenic and/or toxic viral genes. Because the vector is ultimately responsible for the transfer of genes to the fetus, the choice of vector is of utmost importance in fetal gene therapy. Without delving into a complete discussion of viral vectorology, which is well beyond the scope of this chapter, it is important to note that the specific vector to be used for a given IUGT application will depend largely upon one's goals and the disease and/or cell type being targeted. This vector should be selected after careful consideration of such factors as the ability to integrate into host genomic DNA, tissue tropism, packaging capacity, and potential immunogenicity. Most investigators in the field would likely agree that an ideal vector for curing a genetic disease via IUGT (or postnatal gene delivery, for that matter) would selectively target a specific cell type/organ and be able to mediate sufficient levels of gene transfer to produce therapeutic effect with only a single application.

To consider initial target diseases for exploring the therapeutic potential of IUGT, it goes without saying that testing for the target disease must be in place to allow accurate diagnosis before birth, and there must be compelling reasons to pursue prenatal treatment rather than waiting until after birth. Using a variety of animal model systems and rodent models of human genetic diseases and a wide range of transduction methods, IUGT has been targeted to multiple organs [128,181,184,189–208], and in several disease models, phenotypic rescue has been

accomplished [128,181,184,194–196,199–201,209–225]. In the interest of space, and to illustrate the profound therapeutic potential of IUGT, and the ease with which it could be implemented clinically to cure disease, the next section of this Chapter will focus on hemophilia A (HA), presenting HA as an archetype for the host of genetic diseases that could be corrected by IUGT, the rationale for pursuing its treatment prior to birth, the feasibility of doing such, and clinical, societal, and financial advantages IUGT could offer over existing treatments for this disease.

The need for better hemophilia A treatments

HA is the most frequent inheritable coagulation deficiency [226]. The clinical severity of HA is determined by the patient's FVIII plasma levels, but up to 70% of patients with HA present with a severe (<1% of normal FVIII levels in plasma), life-threatening phenotype [227–229]. These patients suffer frequent spontaneous hemorrhaging, which leads to hematomas, chronic painful and debilitating arthropathies, and potentially life-threatening internal bleeding [227]. The current HA standard of care is 2–3 prophylactic intravenous infusions of recombinant or plasma-derived FVIII per week to maintain hemostasis. This "protein-replacement therapy" has greatly improved QoL and extended the life expectancy for many patients with HA. However, it is far from ideal, as it sentences patients to a lifetime of intravenous infusions multiple times each week, and saddles patients with treatment costs that can exceed $300,000/year. Even among the ~25% of HA patients worldwide who are fortunate enough to have access to FVIII prophylaxis, approximately 30% will mount an immune response (inhibitors) to the infused FVIII [230]. In the best-case scenario, these inhibitors reduce the effectiveness of subsequent FVIII infusions; in the worst-case scenario, they cause treatment failure, putting the patient at risk of a life-threatening bleed. These significant shortcomings highlight the need for novel therapies that can provide longer lasting correction, or permanent cure, of HA.

In contrast to current protein-based therapeutics, a single successful gene therapy treatment could promise lifelong correction of HA; indeed, several aspects of HA make it a unique and ideal target disease to correct with gene therapy [206,231–239]. First, although the liver is thought to be the major site of FVIII production within the body [240], FVIII need not be expressed in either a specific tissue or cell type to produce a therapeutic effect. As long as it is produced by cells that have access to the circulation, FVIII can exert its appropriate clotting activity. Second, even if FVIII levels could be restored to only 3%–5% of normal, this seemingly minimal change would exert a marked clinical effect and greatly improve the QoL of patients with severe HA, converting these patients to a moderate/mild phenotype. Conversely, even supraphysiologic FVIII levels are predicted to be safe, creating a wide therapeutic window for HA gene therapy [229]. Armed with this knowledge, the hemophilias were listed among the most promising, "Target 10" diseases in the roadmap that the American Society of Gene and Cell Therapy (ASGCT) (www.ASGCT.org) provided to NIH director, Dr. Francis Collins.

Preclinical animal models for hemophilia A and recent clinical successes

A number of animal models have been developed to evaluate new methods of not only treatment of coagulation disorders but also the prevention and treatment of inhibitor formation. Fortunately, colonies of HA dogs in which spontaneous mutations occurred within the FVIII gene [241,242] and FVIII-deficient mouse models produced via gene targeting and knockout technologies [243] are both available to study the basic biology and function of FVIII and to explore/develop gene therapy–based approaches for treating HA. Pronounced therapeutic benefit has been demonstrated in multiple studies postnatal gene therapy studies in murine models [234,236,244–250]. Phenotypic correction has also been achieved with postnatal gene therapy in dogs with HA, but correction in this more clinically predictive model has proven far more difficult than in mice [251,252].

Despite the promising results obtained in both these models, however, until recently, no therapeutic benefit had been seen in any of the postnatal clinical gene therapy trials conducted for HA, which was in marked contrast to successes that had been reported in clinical gene therapy trials treating patients with hemophilia B (HB) [253]. The past 2 years, however, have seen unprecedented progress in the successful clinical use of postnatal gene therapy to treat HA. Spark Therapeutics recently presented [254] results of a Phase 1/2 Trial [ClinicalTrials.gov Identifier: NCT03003533] testing the efficacy of their proprietary AAV-based gene therapeutic, Spk-8011, in 12 adult HA patients. The results of this trial thus far have been highly promising, demonstrating durable FVIII expression, a 94% reduction in bleeds, and a 95% reduction in FVIII infusions. However, two of the patients developed a significant immune response to the AAV capsid, one of which required hospitalization. This anticapsid response, and the steroid therapy administered to counter it, led to a drop in FVIII levels to below 5% in these patients [254].

Bayer also recently initiated a Phase 1/2 open-label safety and dose-finding study of their AAV therapeutic,

BAY2599023 (DTX201), which has the hu.37 capsid, in adults with severe HA [ClinicalTrials.gov Identifier: NCT03588299]. This trial will have 18 participants but began enrolling in November 2018, so no results have yet been reported.

The most exciting clinical findings in an HA gene therapy trial, however, have come from BioMarin [ClinicalTrials.gov Identifier: NCT02576795]. Patients were administered a single IV injection of either a low dose (4×10^{13} vg/kg) or high dose (6×10^{13} vg/kg) of BioMarin's AAV-based HA gene therapy vector, valoctocogene roxaparvovec (BMN 270), and some have now been followed for over 2 years. In the high-dose cohort, patients experienced a 97% reduction in mean annualized bleed rate (ABR), a 96% reduction in mean FVIII usage, no spontaneous bleeds, and elimination of all bleeds in target joints. QoL as measured by the six-domain Haemo-QoL-A instrument rapidly improved across all domains. Throughout the nearly 2-year follow-up in this high-dose cohort, mean FVIII activity levels have been consistently within the normal or near-normal range in all patients, and the mean FVIII activity level of this cohort as a whole is 59%. The low-dose cohort also showed a 92% reduction in ABR, and a 98% decrease in mean FVIII usage. Consistent with the reduction in ABR and FVIII usage, QoL in this low-dose cohort was also markedly improved. At 1-year postvector infusion, the mean FVIII activity level of the low-dose cohort as a whole was 32%.

Overall, this ongoing trial has demonstrated BMN 270 to be fairly well tolerated at all tested doses. Importantly, no participants developed inhibitors to FVIII, and no participants withdrew from the study. The most common adverse events (AEs) across all dose cohorts were as follows, listed with percentage of participants experiencing each AE: alanine aminotransferase (ALT) elevation (73%); arthralgia (60%); aspartate aminotransferase elevation (53%); headache (47%); back pain and upper respiratory tract infection (40%); and fatigue, insomnia, and pain in extremities (33%) [255]. In the high-dose cohort ($n = 7$), the first treated subject exhibited a 1.5-fold increase in ALT over baseline at 4–7 weeks postvector infusion. This was presumed to be due to mounting of an anticapsid immune response, and the patient was placed on steroids. All subsequent patients in the high-dose cohort were treated prophylactically with steroids. Interestingly, this prophylactic steroid therapy did not halt ALT elevation in four of the seven patients in this high-dose cohort, yet no capsid-directed T-cell response was seen in any of these subjects, raising the possibility that the observed ALT elevation may not, in fact, be due to an immune response, but rather to direct hepatotoxicity or other unknown effects, perhaps arising from the use of an insect-line/baculovirus-based manufacturing process. It is also possible that this manufacturing process somehow causes the immune response to AAV-5 to differ from that of other AAV vectors, which are produced in mammalian cells via plasmid transfection [256].

Given these recent successes, gene delivery strategies using AAV vectors are likely to become more mainstream treatment options for HA (and other diseases as well). Despite these successes, however, a major barrier exists to prevent the widespread adoption of AAV-based gene therapy: the high seroprevalence of neutralizing Abs (NAbs) to AAV that exists in the general population. Recent studies suggest that 30%–70% of patients have NAbs to specific serotypes [257–259]. As such, these patients will not be candidates for gene therapy treatments using AAV vectors. Numerous attempts have been made to eliminate preexisting Nabs, including the use of immunosuppression [260,261], plasmapheresis [262], the inclusion of empty capsids to serve as "decoys" [263], novel bioengineered capsids, or localized vector infusion [264]. Sadly, however, these approaches have only modestly decreased titers and have not succeeded in eliminating the NAbs. Performing gene therapy in utero would eliminate this problem, as the fetus lacks Abs to AAV. Moreover, a stable level of FVIII early in life would prevent the onset of joint bleeds and the development of arthropathy.

Sheep as a preclinical model of hemophilia A

To develop and test the efficacy and safety of IUGT approaches to treating HA and ensure that the data generated are predictive of clinical outcome when these therapies are translated to human patients, it is essential to employ a preclinical animal model that both precisely mimics the disease process of HA, and closely parallels normal human immunology and physiology. To this end, we used a variety of reproductive technologies to successfully reestablish, and then clinically characterize, a line of sheep [265–269] that possess a spontaneous frameshift mutation [267,268] that results in severe HA, which, if not treated at birth, is fatal within the first hours/days of life [270–272]. All affected animals born thus far have experienced multiple spontaneous episodes of severe bleeding, including muscle hematomas, hematuria, and hemarthroses, all of which have promptly responded to infusion of human FVIII. Importantly, chromogenic assays performed independently at the Blood Center of Wisconsin and Emory University revealed undetectable FVIII activity in the circulation of these sheep, explaining their severe phenotype. Importantly, just like human patients with severe HA, these sheep experience frequent spontaneous bleeds into their "knees," which, over time, produce crippling arthropathies that ultimately lead to decreased movement, difficulties walking, and eventually symptoms of pain even just to stand up. These recurring spontaneous

joint bleeds make this line of sheep unique among animal models of HA. Also in similarity to human patients, some of these sheep developed inhibitors following FVIII administration. However, since we had not yet cloned and sequenced ovine FVIII, we were restricted to treatment with human FVIII, leaving unanswered the question of whether these animals will also make inhibitors to the ovine protein. An ongoing collaboration with Drs. Spencer and Doering at Emory University has resulted in the successful cloning and large scale production of recombinant B domain−deleted ovine FVIII [273], making it possible to address this important question and to construct gene therapy vectors encoding ovine FVIII for testing in this valuable model.

As discussed in detail in the section on In utero transplantation experiments in large preclinical animal models, sheep possess many characteristics that make them an ideal preclinical model for IUGT. An additional unique advantage to using sheep in the context of HA treatment is that, like humans, the majority of the FVIII carrier protein, vWF, is stored/located within their platelets. This is in contrast to dog, in which vWF circulates free in plasma [274,275]. This key difference makes the sheep the most clinically relevant large animal model in which to test the efficacy of platelet-targeted gene therapy approaches for treating HA [239,276−278]. In addition, we also reported that sheep naturally harbor Abs to many of the serotypes of AAV being employed as gene delivery vectors [279], suggesting they may also represent a unique preclinical model in which to explore means of circumventing this significant clinical hurdle. For these collective reasons, we feel that sheep are an especially fitting model in which to develop and test gene therapy treatments for HA.

In addition to carefully defining the clinical picture of these animals, we also sequenced the entire coding region of the ovine FVIII gene to determine the molecular basis for their disease. In similarity to mutations seen in many human patients [280], these animals possess a premature stop codon with a frameshift mutation. This is the only animal model of HA with this clinically relevant mutation-type, providing a unique opportunity to study therapies in this context. Armed with this genome sequence data, we next developed a polymerase chain reaction (PCR)-based test to enable us to screen for the disease-causing mutation and thereby identify affected animals at birth and even in utero, using amniotic fluid-derived cells [265−269]. We have also recently shown, by immunohistochemical staining of tissue sections with Abs to various epitopes of the FVIII molecule, which these sheep are completely devoid of any FVIII antigens. As such, they are an excellent model of severe, cross-reacting material-negative HA patients and thus represent a highly stringent preclinical system to study the ability of in utero interventions to induce tolerance to FVIII.

Beyond the value of another large animal model of HA and the uniqueness of the mutation, sheep possess many characteristics that make them an ideal preclinical model for gene therapy, especially in the context of HA. First, sheep share many important physiological and developmental characteristics with humans, they have been used extensively in the study of mammalian fetal physiology, and the results obtained with this model have been directly applicable to the understanding of human fetal growth and development. In contrast to other large animal models such as dog and pig, sheep, like humans, typically have singleton or twin pregnancies, rather than large litters of offspring. Second, sheep are fairly close in size to humans, both at birth and as adults, which would likely eliminate the need for scale-up of vector dose to move from experiments in sheep to trials in humans. Third, the development of the sheep immune system has been investigated in detail [103−109], making sheep well suited for studying the immunological aspects of gene therapy for HA. An additional unique advantage to using sheep to study HA treatment is that in sheep, like human, a large percentage of the vWF is found within platelets rather than free in plasma (*Robert Montgomery and Qizhen Shi, personal communication*). This is in contrast to dog, in which vWF circulates free in plasma [274,275], making the sheep an ideal large animal model in which to explore the use of platelet-targeted gene therapy for HA [239,276−278,281−283]. For these reasons, we feel sheep are a particularly relevant model for developing and testing gene-based HA treatments.

Feasibility and justification for treating hemophilia A prior to birth

Even if FVIII costs were reduced to the point that most HA patients could afford prophylaxis, these patients would still require recurrent, intravenous infusions throughout their lives, be plagued by subclinical microbleeds resulting from the troughs in circulating FVIII levels that occur between infusions, and still have a significant risk of treatment failure due to inhibitor induction. These problems, as well as many of the obstacles that have dogged clinical gene therapy trials to-date, could likely be overcome/eliminated by performing gene therapy prior to birth. For individuals with a family history of HA (~75% of HA cases), prenatal diagnosis for HA is feasible, available, and is both encouraged and cost-effective, even when considering developing third-world countries [284−295]. Moreover, recent studies have proven the feasibility of diagnosing HA in utero via digital PCR on the small number of fetal cells present within the mother's blood, enabling prenatal diagnosis of HA with effectively zero risk to the fetus or mother [294,296].

Although most individuals with a family history of HA are encouraged to have prenatal screening, however, parents currently have only two choices following prenatal diagnosis of HA: pregnancy termination or the birth of a child with HA. The availability of a safe and effective in utero treatment would provide parents with a much needed third option, which would certainly provide the needed impetus for much more widespread prenatal HA screening. While in vitro embryo screening and selection has been proposed as a possible solution in families with a history of HA and other genetic diseases, such an approach is very expensive and requires highly skilled personnel and specialized equipment/facilities, which would not likely be available in much of the world. In contrast, IUGT does not require any sophisticated equipment that would not already be in place for prenatal diagnosis, and it should not be prohibitively expensive.

Since parents currently have no therapeutic options for treating a child diagnosed in utero with HA, ~1 in 5000 boys born each year worldwide is affected with HA [226], despite the availability of prenatal screening. In the United States alone, correcting this disease prior to birth could benefit the ~240 patients/year born into families with a history of HA. IUGT could promise the birth of a healthy infant who required no further treatments, removing the heavy physical, psychological, and monetary burden on the patients, their families, and the health care system. The current estimate for the lifetime cost of prophylactic treatment for one HA patient is $20 million. Curative IUGT would thus save ~$48 billion over the lifetime of the HA patients born just this year in the United States. Such a treatment, if successful, would also clearly represent a major advance with respect to the QoL of the patient, as the patient would receive a single treatment rather than a lifetime of expensive and potentially dangerous infusions several times each week.

When considering a prenatal treatment, especially for a disease involving abnormal coagulation, safety of the fetal patient is paramount. Importantly, the fetus possesses unique hemostatic properties that make it an ideal patient. During early fetal life, activation of FX occurs predominantly via tissue factor activity, making it largely independent of the FIXa/FVIIIa phospholipid complex [297]. As a result, the fetus develops without hemorrhage, despite having little or no expression of FVIII and FIX [297–299]. The unique hemostasis of the fetus should thus allow IUGT to be performed safely for HA; indeed, one of the 48 human patients that has thus far received IUTx [181,182] was transplanted in the hopes of correcting HA, or at least inducing immunological tolerance to FVIII [180,183,300]. While only this one HA patient was treated, he suffered no untoward effects as a result of the in utero intervention, he has thus far exhibited reduced severity of disease compared to his siblings, and he (in contrast to his siblings) has not developed inhibitors with FVIII treatment (*J.L. Touraine, personal communication* and [300]). This remarkable case thus provides clinical validation for prior experimental studies demonstrating that exposure to vector-encoded proteins (including coagulation factors) during early immunologic development induces stable immune tolerance [203,204,301,302]. The lifelong tolerance to FVIII induced by an IUGT-based HA treatment should therefore prevent the development of FVIII inhibitors that plague patients treated with replacement therapy [230,303–306]. In this one clinical case, Dr. Touraine relied on the ability of unpurified fetal liver cells to endogenously produce sufficient levels of FVIII, after transplant, to mediate correction. The only partial correction observed in this patient supports the approach of using gene transfer to ensure adequate levels of FVIII are obtained for full phenotypic correction.

Although the clinical and financial advantages of IUGT are compelling, in and of themselves, it is important to acknowledge that there are also features of the fetus that make it a far better gene therapy recipient than the adult [128,307,308]. For instance, cell populations that are quiescent in the adult, and largely refractory to transduction with many commonly employed viral vectors, are actively cycling in the fetus and amenable to transduction at relatively high efficiencies. For example, we showed that by administering a single IP injection of a small volume of a γ-retroviral vector at the optimal stage of gestation (which we determined experimentally), it is possible to achieve gene transfer levels within the hematopoietic system of 5%–6% [196,222,309], levels that would undoubtedly be beneficial in HA. Further studies involving Ab selection of CD34 + cells and serial transplantation/repopulation [196,222,310], provided compelling evidence that this approach successfully modified bona fide HSC, indicating this method could provide lifelong disease correction.

Our results also demonstrated that this approach successfully transduced hepatocytes and hepatic endothelium at levels that could well be therapeutic in HA, and defined the temporal window during gestation for optimal transduction of these cells [194]. Concurrently, fetal gene delivery experiments conducted in sheep, rodent, and NHP models, by other investigators who employed a variety of viral-based vectors, produced similar results [128,181,184,189–208]. The collective results of these studies clearly support the ability of this method to deliver an FVIII transgene to the nascent liver with sufficient efficiency to convert severe HA patients to a moderate or, perhaps, even mild phenotype [194].

While the active cell cycling in the fetus enables efficient transduction with vectors that require mitosis, it is important to note that this ongoing proliferation in all of

the fetal organs is also of benefit when using vectors that do not have an absolute requirement for mitosis. Gene delivery early in gestation, regardless of the vector employed, also makes it possible to achieve subsequent expansion of these gene-corrected cells throughout the rest of gestation. As such, even if the initial gene transfer only transduces a small number of the desired target cells, this subsequent expansion could produce clinically useful levels of gene-correction by birth.

As mentioned earlier, one of the biggest obstacles/drawbacks to treating severe HA by repeated protein infusion is the formation of inhibitory Abs in ~30% of patients. It is important to note that the distinct immunologic benefits to performing IUTx (which we discussed at length earlier in this chapter) also apply to IUGT. We have spent the last two decades performing IUGT studies in the sheep model [128,194–197,222,280,301,309,311–313], and have shown that it is possible to take advantage of this unique temporal window of relative immuno-naïveté to efficiently deliver exogenous genes a variety of fetal tissues and induce durable tolerance to the vector-encoded gene product [301]. This tolerance induction appears to involve both cellular and humoral mechanisms, since Ab and cellular responses to the transgene product were both significantly diminished in these animals, even several years after IUGT. Further mechanistic studies demonstrated that IUGT early in fetal development exploits both central and peripheral tolerogenic avenues in the fetus [302]. These results strongly imply that IUGT, even if it not curative, would still be an ideal treatment modality for HA, since the induced immune tolerance would ensure that postnatal therapy, be it protein- or gene-based, could proceed safely without any of the immune-related problems that currently plague HA treatment.

Interestingly, although the incidence of HA is ~7 times that of HB, to-date, the only experimental studies to directly investigate IUGT for treating the hemophilias have targeted HB [factor IX (FIX) deficiency] [189,190,202–204,206–208,314,315]. The choice to target HB rather than A most likely results from the greater ease with which FIX can be cloned into a variety of viral vectors, and efficiently expressed upon transduction of appropriate target cells; this is in marked contrast to the difficulties that were initially seen when attempting to express FVIII in the context of viral vectors [316]. The treatment of HB by IUGT has been extensively studied in murine models with gene transfer performed at various gestational ages, via different routes of injection, and using different vector types. Schneider et al. compared IP, intramuscular, and intravenous injections of human FIX carried by adenovectors and AAV-2 into mouse fetuses and found that adenovectors resulted in initially higher levels of FIX. Interestingly, given their episomal nature, adenovector-injected mice maintained therapeutic levels of FIX for 6 months, and no Abs developed against either vector or transgene [208]. In other studies, Sabatino et al. reported low-level human FIX expression following intramuscular injection of fetal and neonatal mice with either AAV-1 or AAV-2. Curiously, the injection of AAV-1 induced tolerance and allowed the postnatal readministration of the FIX-encoding AAV-1 vector, increasing FIX levels sufficiently to reach the therapeutic range, while injection of AAV-2 did not induce immune tolerance [317].

Without a doubt, the most impressive and clinically promising results of IUGT in hemophiliac mice were achieved by Waddington et al., who injected a FIX-encoding lentiviral vector into E15 mouse fetuses and demonstrated therapeutic levels of FIX (9%–16% of normal) and improved coagulation for 14 months post-IUGT. Furthermore, no immune response developed to FIX, even when the protein was repeatedly injected postnatally [204].

Collectively, these murine studies have provided compelling evidence that IUGT can result in expression of FIX at levels that not only have therapeutic significance, but are often sufficient to induce tolerance, thus allowing postnatal administration of the same vector or the FIX protein without eliciting an immune response. Because HA patients have at least a 10-fold higher likelihood of developing inhibitors than HB patients [318,319], these studies, while encouraging, leave unanswered the critical question of whether fetal gene delivery's ability to induce immune tolerance to marker gene products and FIX will hold true for the induction of tolerance to FVIII, given FVIII's higher inherent immunogenicity. What we are currently addressing is an important question in the sheep model.

All of the afore-referenced studies demonstrated that the direct injection of viral vectors into the developing fetus can be an effective way of delivering an exogenous gene and achieving long-term expression in multiple tissues and confirmed the therapeutic potential of an in utero approach to gene therapy. However, for this technology to move forward into the clinical arena will likely require the development of vectors that can target specific cell types following in vivo administration, to eliminate the risk of inadvertently modifying nontarget cells, like those of the germline [192,193,280], following a direct vector injection approach to IUGT. In an effort to develop safer means of correcting HA and other diseases prior to birth, we have been exploring the possibility of using mesenchymal stromal cells (MSC) as vehicles to deliver exogenous genes to the developing fetus. In the next section of this Chapter, we discuss our rationale for using these cells as therapeutics for HA and summarize our promising results using FVIII-expressing MSC to treat pediatric HA sheep.

Mesenchymal stromal cells as hemophilia A therapeutics

Decades after the groundbreaking studies that Friedenstein performed to better understand the marrow microenvironment [320,321], results from various labs around the world have revealed that MSC possess a very broad differentiation potential, both in vitro and in vivo and exhibit properties that suggest at least a subset of this population may be true stem cells [93–95,322–331]. MSC are very rare, only comprising roughly 0.001%–0.01% of cells within the marrow [332]. However, they can be passaged extensively in vitro without a loss of differentiative potential, making it possible to extensively expand these cells in vitro to easily obtain sufficient numbers for clinical use [333]. Since MSC were first discovered within the BM, much of the work to-date has focused on MSC isolated from this tissue. However, we and others have now shown that cells with the phenotype and functionality of MSC can also readily be isolated from numerous tissues, including brain, liver, lung, fetal blood, umbilical cord blood, kidney, and even liposuction material [92,334–340]. Importantly from the standpoint of in utero therapies, MSC have also been isolated from the amniotic fluid and the chorionic villi, raising the exciting possibility that autologous MSC could be used as cellular therapeutics or gene delivery vehicles for in utero therapy [341–346].

As discussed earlier, the liver is believed to be the primary anatomic site of FVIII synthesis/production. Studies from our group and others over the past decade have provided compelling evidence that MSC from various sources can generate, in vitro and in vivo, cells which are indistinguishable from native hepatocytes, and have shown that transplantation of MSC in a range of model systems results in fairly robust formation of hepatocytes which repair a variety of inborn genetic defects and injuries [93,94,97,98,100,327,347–371]. Of direct relevance to HA treatment, we have found that, after transplantation into fetal sheep, human MSC engraft at levels of up to 12% within the recipient liver [93,98,100,323,324,372–375] and contribute to both the parenchyma and the perivascular zones of the engrafted organs, positioning them ideally to deliver FVIII into the circulation. Since FVIII levels of 3%–5% of normal would convert a patient with severe HA to a moderate or mild phenotype, it seems reasonable to conclude that these levels of engraftment produce a therapeutic benefit. In other recent studies, we have demonstrated that MSC from various tissues throughout the body endogenously produce and secrete biologically active FVIII [376]. Collectively, these results suggest that MSC are ideally suited for treating HA.

However, upon further analysis, we found that, although MSC engrafted at significant levels within the natural sites of FVIII synthesis, the levels of FVIII production were too low to provide an effective means of treating HA. By using gene therapy to engineer MSC to express FVIII, however, it is likely that the levels of engrafted MSC we have thus far achieved in utero could provide therapeutic benefit in HA, especially if newer, expression-optimized FVIII variants were employed [233,250,377,378]. Importantly, MSC are efficiently transduced with all of the major clinically prevalent viral vector systems including adenovirus [379–381], murine retroviruses [381–385], lentiviruses [386–391], and AAV [392,393]. Furthermore, in contrast to studies with hematopoietic stem cells [394–396], human MSC are stable in culture, do not form tumors in vivo (unlike murine MSC [397]), and there is no evidence that human MSC transform or progress to clonal dominance following transduction. In fact, even following intentional induction of genomic instability, human MSC have been shown to undergo terminal differentiation rather than transformation [398], with very rare transformants only arising after very extended in vitro propagation, and being easily identifiable (and removable) based on their altered cell surface marker profile [399]. As such, MSC appear to represent very safe cellular vehicles for delivering a therapeutic gene.

Looking specifically at using MSC to treat HA, multiple studies have already proven that MSC can be efficiently transduced with murine retroviral and lentiviral vectors with gene cassettes encoding FVIII from a variety of species and secrete high levels of functional FVIII protein. Importantly, FVIII purified from the conditioned medium of the transduced MSC had a specific activity, electrophoretic mobility, and proteolytic activation pattern that was identical to commercially produced FVIII [400]. Given the widespread distribution and engraftment of MSC following transplantation, the ability of MSC to give rise, in vivo, to cells of numerous tissue types, and their ability to efficiently process and secrete high amounts of biologically active FVIII, it is not surprising that we and others feel that MSC represent ideal vehicles for delivering an FVIII transgene throughout the body, and thereby providing long-term/permanent correction of HA [400–403].

In addition to their widespread engraftment and their ability to serve as delivery vehicles for the FVIII gene, MSC have rather unique immunological properties that may further increase their utility for treating HA. MSC do not normally express MHC class II or the costimulatory molecules CD80 and CD82, and as such, they do not provoke the proliferation of allogeneic lymphocytes or serve as very effective targets for cytotoxic T- or NK cells. In fact, a large body of evidence is now accumulating that MSC can be transplanted across allogeneic barriers without eliciting a pronounced immune response [404,405]. Thus off-the-shelf MSC from an unrelated donor could

theoretically be used to treat HA, greatly increasing the feasibility of obtaining and using these cells for therapy. MSC also possess the clinically valuable ability to selectively migrate to sites of injury and/or inflammation within the body, whereupon the MSC then mediate repair both by engrafting and generating tissue-specific cells within the injured tissue [406–408], and by releasing trophic factors that blunt the inflammatory response and often promote healing by activating the tissue's own endogenous repair mechanisms. This property raises the exciting possibility that, following infusion, FVIII-expressing MSC might have the ability to selectively migrate to sites of active bleeding/injury, thereby focusing the therapy where it is most needed.

Preclinical success with mesenchymal stromal cell–based hemophilia A treatment

Despite the many physiological and clinical advantages to intervening prior to birth, there are already ~16,000 people in the United States alone with HA, who obviously would not benefit from the development of an in utero therapy. Furthermore, over 25% of HA-causing mutations arise de novo, making it unlikely this patient population would be screened prenatally. We therefore began investigating, in two pediatric HA lambs, whether the many advantages of MSC as a cellular vehicle for delivering an FVIII gene can still be realized if these FVIII-expressing MSC are transplanted during early childhood. During the first 3–5 months of life, both these animals had received frequent, on-demand infusions of human FVIII for multiple hematomas and chronic, progressive, debilitating hemarthroses that had resulted in severe defects in posture and gait and rendered them nearly immobile. Given the severe, life-threatening phenotype of the HA sheep, we elected to utilize haploidentical allogeneic cells from the ram that had sired the two HA lambs, rather than attempting to collect marrow to isolate autologous MSC.

Based on our prior in utero studies, we knew that MSC should engraft throughout all of the major organs [93,94,98,409–411] and durably express the vector-encoded genes [94,410,412] following IP injection. The IP route also enabled the MSC to enter the circulation in an almost time-release fashion, as they were absorbed through the peritoneal lymphatics. The IP route also allowed us to avoid the extensive lung-trapping which occurs following IV administration of MSC, promising more efficient delivery of the MSC to the desired target tissues and eliminating the clinical risk of emboli formation [413,414].

MSC were simultaneously transduced with two lentivectors: the first encoded an expression/secretion optimized porcine FVIII (pFVIII) transgene [415] and the second encoded an eGFP reporter to facilitate tracking of donor cells in vivo. Because the pFVIII transgene had previously been shown to be expressed/secreted from human cells at 10–100 times higher levels than hFVIII [233,234,378], we reasoned that even low levels of engraftment of the transduced MSC should exert a therapeutic benefit. Once the transduced MSC were sufficiently expanded, they were transplanted via IP injection, under ultrasound guidance, in the absence of any preconditioning.

Following transplantation, a highly sensitive chromogenic assay was unable to detect any FVIII activity in the circulation, but the clinical picture improved dramatically in both animals. All spontaneous bleeding events ceased, and they enjoyed an event-free clinical course, devoid of spontaneous bleeds, obviating the need for hFVIII infusions. Even more remarkably, existing hemarthroses resolved, their joints recovered fully, and they regained normal posture and gait, resuming a normal activity level. To our knowledge, was the first report of phenotypic correction of severe HA in a large animal model following transplantation of cells modified to express FVIII, and the first time that reversal of chronic debilitating hemarthroses was achieved in any setting.

Upon euthanasia, PCR analysis demonstrated MSC engraftment in nearly all tissues analyzed, including liver, lymph nodes, intestine, lung, kidney, omentum, and thymus. Subsequent confocal analysis on frozen tissue sections revealed large numbers of FVIII-expressing MSC within the synovium of the joints which exhibited hemarthrosis at the time of transplant, confirming the intrinsic ability of transplanted MSC to home to and persist within sites of ongoing injury/inflammation, releasing FVIII locally within the joint, and providing an explanation for the dramatic improvement we observed in the animals' joints. Collectively, the results of the PCR and confocal analyses strongly support the conclusion that widespread durable engraftment of MSC can be achieved in a large animal model following transplantation in a postnatal setting, without the need for preconditioning/ablation. However, the levels of engraftment seen in these pediatric animals were substantially lower than those obtained in our prior in utero studies. Moreover, despite the marked clinical improvement and the widespread engraftment of the transplanted MSC, both animals mounted a fairly robust immune response to pFVIII, in similarity to prior studies performed with HA mice [378]. These inhibitors exhibited cross-reactivity to hFVIII, which was surprising, given the well-established ability to use pFVIII products in human patients with existing anti-hFVIII inhibitors [416–419].

Therefore this postnatal study proved that MSC can serve as cellular vehicles to deliver FVIII and produce a therapeutic benefit, and we posit that administering this

same treatment in utero would have a more pronounced and more durable effect, since higher levels of donor MSC engraftment could be achieved, and because inhibitor formation could be avoided due to the induction of immune tolerance to the FVIII transgene.

Risks of in utero gene therapy

Despite the great promise IUGT holds for the treatment of HA and the myriad other genetic diseases that can be diagnosed prenatally, several important safety concerns must be addressed prior to its clinical application. While the risks of postnatal gene therapy have been recognized and extensively discussed, specific risks may be higher for the fetus than for the postnatal recipient. There are two sets of potential safety concerns associated with IUGT: those associated with fetal intervention, and those due to the gene transfer itself. As with any fetal intervention, infection, preterm labor, and fetal loss are all theoretically possible. In reality, however, a wealth of clinical data exist that provide unassailable proof that the early human fetus can be accessed multiple times with an extremely low procedure-related risk, assuming that a minimally invasive, ultrasound-guided approach is employed [62,75,156,176,180,181,184].

The risks that cause the most concern regarding the use of IUGT include disruption of normal organ development, insertional mutagenesis, and germline transmission [420]. Although IUGT holds great potential for restoring normal function, manipulating the fetus has the potential to alter normal organ development, and the possibility for deleterious effects due to the injection and from any inherent toxicity of the vector itself both need to be considered and carefully evaluated. NHPs injected with lentiviral vectors in utero via either the intrapulmonary or intracardiac route showed no adverse effects on postnatal heart and lung development [200]. In contrast, studies performed by Gonzaga et al. found that expression of FGF-10 in the developing rat lung following IUGT leads to cystic adenomatoid malformations illustrating how the forced expression of a specific transgene can lead to malformation [421]. These findings suggest that strategies involving expression of growth factors, transcription factors, or other regulatory molecules will need to be carefully examined, as they may have significant potential to alter normal organ development, particularly early in gestation.

Genomic integration–associated insertional mutagenesis

Insertional mutagenesis is a major concern with all of the integrating viral vectors and has been the subject of intense investigation since the clinical observation of four cases of T-cell leukemia, diagnosed 31–68 months after postnatal γ-retroviral-mediated gene transfer to autologous HSC to correct children with X-linked SCID. This concern was further heightened when linker-mediated PCR analysis of lymphocytes from these patients revealed that insertional mutagenesis had occurred in all four cases, and was at least partially responsible for the observed leukemogenesis [422–424]. Importantly, in our long-term IUGT studies in fetal sheep, we also employed γ–retroviral vectors and achieved significant levels of gene transfer to hematopoietic cells, which persisted in these sheep throughout the 5-year course of study [196,222,309]. Moreover, transgene-positive CD34 + cells could be detected in the marrow of these animals several years post IUGT [310], and gene-marked BM cells isolated from these IUGT recipients were able to serially engraft secondary fetal sheep recipients. These three pieces of data demonstrate that this approach resulted in gene transfer to *bona fide* HSC, yet we never observed leukemogenesis in any of these animals. Given that sheep have a lifespan of roughly 10 years, this study should more or less approximate a 35-year follow-up in "human years". The difference between our study and the clinical trial (aside from the obvious species difference) that likely explains the differing outcome is the differing transgene. In our experimental proof-of-concept studies, we employed marker genes to facilitate tracking and quantitation of gene-marking in various tissues. In the clinical trial for X-SCID, the vector encoded the therapeutic common gamma chain (γc) gene (IL-2RG), as this was the gene defect causing X-SCID. Subsequent studies revealed that the observed leukemogenic event in these patients was likely the result of a combinatorial effect of both the insertion of the vector in close proximity to the LMO-2 gene (which has, itself, been associated with T-cell leukemias) and a growth advantage conferred on the transduced cells by the high expression levels of the therapeutic γc gene [425–428]. Further adding to the concerns regarding insertional mutagenesis is a study recently presented at the 2019 ASGCT meeting in which the authors describe the occurrence of oncogenesis in an X-linked CGD patient treated with γ-retroviral-modified HSC [429]. This study represents the first report of the occurrence of G to A hypermutation occurring during the processes of reverse transcription and integration leading to altered expression of a neighboring cellular gene and oncogenesis. This most recent report provides a sobering lesson in the complexities of gene therapy risk assessment, which is still a relatively new and rapidly evolving field.

Looking specifically at IUGT, to-date there has been only one report of oncogenesis after prenatal gene delivery. In their study, Themis et al. reported a high incidence of postnatal liver tumors in mice following prenatal

injection with a third-generation equine infectious anemia virus (a lentivirus) vector. These tumors were not seen in mice that received a very similar vector constructed on an HIV backbone [430]. The authors did not identify the genomic insertion sites in these animals, so it remains unclear whether insertional mutagenesis was the cause of the observed tumor formation. Nevertheless, this important study demonstrates that the fetus may be particularly sensitive to tumorigenesis induced by certain vectors.

In another recent study, Chan et al. made the surprising finding that long-term expression of vector-encoded coagulation factors IX and X following liver-directed IUGT with AAV-5 or AAV-8 vectors in NHP fetuses at ~0.4 gestation was largely due to the genomic integration of the AAV vectors within the genome of the recipients' hepatocytes. Specifically, 57%–88% of AAV sequences that were retrieved from recipient hepatocytes exhibited random genomic integration of the AAV genome [431]. Despite several reports to the contrary [432,433], it has long been the accepted dogma that AAV vectors integrate only very rarely, if at all, into the host genome following transduction [434,435]. As such, these findings are particularly surprising and troubling from a safety standpoint, as they suggest that insertional mutagenesis may also be a possibility with AAV vectors. Indeed, two studies in murine models have provided evidence that AAV integration in hepatocytes can, under the right conditions, lead to oncogenesis [436,437]. Collectively, these experimental and clinical findings suggest that preclinical assessment of the risk of insertional mutagenesis following IUGT will require very carefully designed studies with the actual vector to be employed for the pending clinical trial, in an animal model that has been thoroughly validated in the setting of the target disease.

Potential risk to fetal germline

While gene transfer to the vast majority of the fetal tissues would be desirable for correcting diseases, such as the hemophilias, that would benefit from widespread systemic release of a secreted transgene product, PCR analyses of sheep that had received IUGT also revealed that the fetal reproductive tissues often contained the vector sequences, raising the troubling possibility that the vector may have reached the developing germline [196]. In the human fetus the primordial germ cells (PGC) are compartmentalized in the gonads at 7 weeks of gestation [438]. As such, the germline should only be accessible through the vascular system, and targeted gene therapy that is administered after this time period should not affect the germline. Nevertheless, the possibility of inadvertent germline gene transfer is clearly a major safety concern and a bioethical issue, and as such, this critical issue needed to be examined in greater detail.

Since prior studies had demonstrated that both the embryonic germline [439–442] and isolated PGC [443] can readily be infected with γ-retroviral vectors and pass the vector genetic material to subsequent generations in a Mendelian fashion as part of the permanent genome, we employed a three-tiered approach to scrutinize this important issue in detail in the sheep model of IUGT: (1) tissue sections prepared from the in utero treated animals were examined by immunohistochemical staining; (2) we performed genetic analysis on isolated sperm cells from the treated males; and (3) we conducted breeding experiments in a limited number of animals and analyzed the resultant offspring [193,195,196,222]. These studies indicated that although the fetal ovaries appeared to be unaffected by this approach to IUGT, numerous cells within the developing fetal testes were modified, including interstitial cells, Sertoli cells, and small numbers of both immature germ cells within the forming sex cords and the resultant sperm cells.

Importantly, however, gene-modified germ cells were only observed in two of the six animals examined in our studies, and, in these two animals, the incidence of germ cell modification was well below the theoretical level of spontaneous mutation within the human genome [444]. This low frequency of modification coupled with observations that genetic alterations to the germ cells may produce deleterious effects, placing them at a disadvantage during fertilization, suggest that the likelihood of any genetic alterations present being passed to subsequent offspring would be extremely unlikely. In agreement with this supposition, we did not observe transfer of the vector sequences in any of the 10 offspring we studied, even when both the parents had received IUGT. This is clearly an issue that will need to be addressed in greater detail, nevertheless, prior to moving IUGT into clinical trials. This need for further investigation is underscored by the fact that, in other studies employing lentiviral vectors in NHPs, Tarantal et al. observed modification of the female germline, but no effect upon the male germ cells [192]. Thus the issue of germline safety will likely have to be investigated in more than one preclinical model, employing the specific vector being considered for clinical use, in order to obtain an accurate assessment of the risk posed by the procedure.

While these studies in different animal models both suggest that the frequency of germline transduction is low and related to gestational age and mode of vector administration, they also suggest that low-level transduction of germ cells after systemic administration of integrating vector to the fetus may not be entirely avoidable. As such, when contemplating ultimate clinical application of IUGT, careful consideration may need to be given to

determining what frequency of potential germline transduction is considered acceptable in the context of treating a severe, perhaps life-threatening genetic disorder.

Conclusion and future directions

Although great progress has been made, there are many remaining hurdles for IUTx and IUGT to overcome before they become mainstream clinical modalities. Challenges for IUTx are primarily related to overcoming the competitive barriers to engraftment in the fetus, and better defining the innate and adaptive immune limitations to engraftment in large animals and humans. As our understanding of stem-cell biology and the ontogeny of hematopoiesis and the hematopoietic niche ontogeny advance, the therapeutic applications of IUTx will likely expand from their current narrow focus to include the treatment of nonhematopoietic diseases. While the strategy of prenatal tolerance induction for facilitation of postnatal HSC transplantation is nearing clinical application and has great potential to benefit many patients, the development of an IUTx strategy that allowed a single-step treatment to achieve therapeutic levels of engraftment would be ideal, and it would likely propel this promising therapy into the clinic.

IUGT holds even greater promise for treating/curing essentially any inherited genetic disease. From our findings in the sheep model and those of other groups exploring IUGT in sheep, mice, and NHPs, it is clear that the direct injection of viral vectors into the developing fetus can be an effective way of delivering an exogenous gene and achieving long-term expression in multiple tissues, suggesting IUGT may one day be a viable therapeutic option for diseases affecting any of the major organ systems. Moreover, even if not curative, IUGT would be ideal for a disease like HA, since lifelong immunologic tolerance could be induced to FVIII, thus overcoming the immune-related hurdles that currently hinder postnatal treatment of this disease.

Given its great potential, the International Fetal Transplantation and Immunology Society recently facilitated a panel discussion of international experts to consider the scientific, clinical, and ethical issues related to prenatal gene transfer for the treatment of genetic diseases. The goal of the session was to revisit the 1999 NIH Recombinant DNA Advisory Committee Policy Conference recommendations [445], examine the current status of IUGT, identify gaps in knowledge in need of resolution, achieve an agreement among the members of the international scientific community regarding target diseases, procedural- and vector-related safety considerations, and define a roadmap (which was recently published [446]) for developing clinically translatable IUGT therapeutic approaches. This discussion was prompted, in part, by two recent preclinical studies that have shown the remarkable therapeutic potential of IUGT and have validated the advantages to correction prior to birth. In the first of these studies [447], Massaro et al. demonstrated the first successful application of IUGT in a mouse model of acute neuronopathic Gaucher disease (nGD). nGD is a human genetic disease in which marked irreversible neurological pathology already manifests in utero. The acute childhood form of nGD is lethal and is untreatable, since enzyme cannot cross the blood–brain barrier. In this remarkable study, the authors showed that IUGT with an AAV vector encoding glucocerebrosidase (GC) reconstituted neuronal GC expression, abolished neurodegeneration and neuroinflammation, and enabled the mice to be fertile, fully mobile, and to live for up to at least 18 weeks. In a second recent landmark study [448], Chan et al. reported that in utero delivery of AAV-5 or -8 vectors encoding human coagulation factor IX (hFIX) or X (hFX) under the control of a liver-specific promoter into NHP fetuses at $\sim$0.4 gestation resulted in sustained curative levels of hFIX and hFX and induced durable immune tolerance to the coagulation factors, in the absence of clinical toxicity, supporting IUGT's therapeutic potential for early-onset monogenic disorders.

After extensive deliberation the panel agreed that IUGT, using an ex vivo or in vivo direct approach, remains the best therapeutic strategy for treating, and ideally curing, a wide range of genetic disorders early in gestation, such as hemophilia, lysosomal storage disorders, and spinal muscle atrophy. The panel also set forth several criteria they felt should be met in order for IUGT to be considered. The first of these was that reliable prenatal enzymatic [449] or genetic diagnosis, and a strong genotype/phenotype correlation impacting clinical prognosis must exist for the disease being considered. Second, it was stressed that there must be a compelling reason to use IUGT, based on a need to (1) intervene during development to prevent damage caused by the disease; (2) make use of unique physiologic characteristics of the fetus, such as the more permissive blood–brain barrier, which make possible successful delivery to otherwise difficult-to-access tissues; or (3) exploit the immunologic environment at the time of treatment to prevent pre- or postnatal immune reaction to the transgene-encoded protein. It was agreed that to be considered as a good candidate for treatment by IUGT, said disease should (1) result in severe fetal morbidity and/or mortality either in utero or postnatally; (2) exhibit poor clinical outcome with existing postnatal therapies, be limited by huge economic burden, or be hindered by immunologic responses/rejection; or (3) not be associated with other serious abnormalities or disorders that are not corrected by the therapeutic gene.

The panel also acknowledged that, as with any fetal intervention, infection, preterm labor, and fetal loss are all

theoretically possible. However, a considerable amount of clinical data exist that provide unassailable proof that the human fetus can be accessed multiple times with an extremely low procedure-related risk. Indeed, the technical aspects pertaining to systemic injections using fetal umbilical vein access or IP injection are safe, straightforward, mainstream procedures [450]. Maternal safety was recognized to be a critical consideration for IUGT, particularly with respect to possible exposure to the viral vectors or gene products infused into the fetus. Although the procedure has proven to pose minimal risk to the mother, it was stressed that infection and impairment of future ability of the mother to reproduce should be considered, as should the possible immunological consequences/risks to the mother as a result of exposure to a viral vector and/or its encoded protein.

It was agreed that many of the concerns and/or limitations that were identified may well be solved in the future with new technologies that allow nonviral delivery, and/or specific cell targeting and gene-editing to minimize the risk of off-target events, or by the use of an ex vivo approach to IUGT.

Brief mention was also made of how the development of gene-editing technologies such as zinc-finger nucleases, TAL effector nucleases, and Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein (CRISPR/Cas) [451,452] has the potential to revolutionize the whole way in which gene therapy is conceptualized. The ability to modify a chosen sequence in its native genomic locus offers incredible advantages, in terms of both safety and efficacy, over current "gene-addition," and would completely eliminate existing concerns related to random genomic integration, inappropriate levels or tissue distribution of transgene expression, and inadvertent germline alteration. As such, these newer technologies are likely to be a key component of future IUGT studies/trials. Indeed, recent work from the Peranteau group has provided compelling proof-of-principle for the ability to effectively use genome-editing technology to correct mutations in metabolic genes and in genes related to monogenic lung disease prior to birth in mice [453,454]. These groundbreaking studies thus provide a glimpse into the exciting future of IUGT and its vast potential to correct genetic diseases.

In closing, IUTx and IUGT hold great promise for treating/curing many inherited genetic diseases. At the present time, these in utero interventions stand at a critical juncture [186] and have vast potential for dramatically improving human healthcare. Many of the most daunting obstacles have recently been overcome in animal models, or are at least better understood, which has reinvigorated this exciting field. Looking specifically at IUGT, gene delivery technologies have evolved significantly in the last decade, such that many of the most daunting obstacles have now better been identified and this exciting field has been reinvigorated. There is no doubt that surpassing the few remaining hurdles to allow clinical implementation of these therapies will dramatically change the whole paradigm for the way we perceive and treat many genetic disorders.

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Chapter 22

Challenges in the development of immunoisolation devices

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Introduction

Cell-based therapies can be used to treat a variety of human diseases. One important class of cell-based therapies is the system where the cells are vehicles for the delivery of therapeutic proteins [1,2]. They are particularly useful in patients who are unable to produce proteins themselves, where they can be used as an alternative to the frequent administration of exogenous protein. For example, many patients with type I diabetes patients inject daily doses of the protein hormone insulin to attempt to normalize blood sugar levels. Unfortunately, insulin therapy is very labor intensive, requiring multiple measurements and interventions per day [4]. In addition, even the best delivery systems do not provide complete control of blood sugar [5]. In contrast, transplanted islets produce insulin in a dynamic and glucose-dependent manner and can provide long-term control of blood sugar [6–8].

Cell-based therapies have been developed with autologous, allograft, or xenograft donor cell sources. In cases where foreign nonself donor cells are transplanted into hosts, immunosuppression is required to prevent immune rejection of the transplanted cells [9–11]. However, these drugs are associated with health risks such as lymphoma and infection, which limits the patients that are suitable to receive these therapies.

An alternative strategy is to use an immunoisolation device. Immunoisolation devices, originally described by Cheng [12], use a barrier to restrict the entrance of host immune cells that can destroy the foreign, donor cells while allowing for the passive diffusion of the therapeutic protein and nutrients/waste products out of the device (Fig. 22.1). Immunoisolation devices have been investigated as a means to protect cells for treatment of a variety of diseases, especially those characterized by a deficiency in the natural production of a specific protein. These include endocrine disorders such as diabetes [13] and hypothyroidism [14]; genetic diseases such as lysosomal storage disorders [15] and hemophilia/anemia [1]; and neurological disorders such as Parkinson's disease [16], Huntington's disease [17], and retinal degeneration disorders [2,18].

In this chapter, we review (1) nutrition of cells within the device, (2) avoidance of the immune system, and (3) engineering approaches to promote viability and functionality of the encapsulated cells. This includes a brief overview of the mechanisms of immune rejection of foreign tissue and an introduction to how this knowledge can be leveraged in the design of immune isolation barriers. We will also discuss the type of cells that can be included in immunoisolation devices and the challenges associated with providing enough nutrition for those cells. Recent advances in current devices will be examined, and finally, an in-depth look into the strategies to prevent biomaterial-mediated host-rejection of immunoisolation devices through fibrosis will be explored.

Rejection and protection of transplanted cells and materials

The host immune system has a number of strategies to defend itself against "nonself" materials, cells, and

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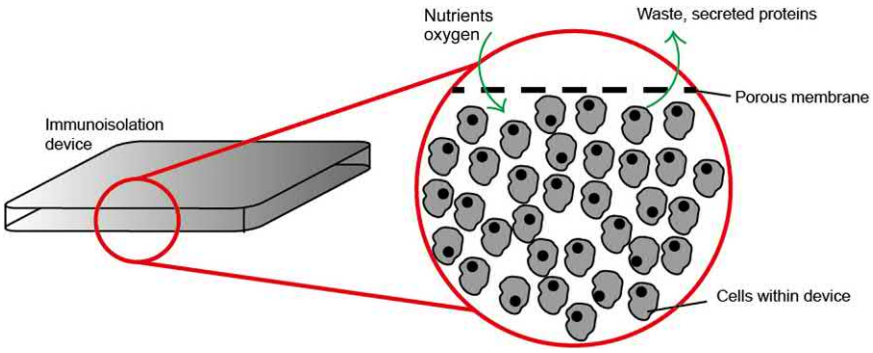

FIGURE 22.1 A schematic overview of an immunoisolation device: cells or clusters of cells are supported in a matrix and entrapped within a device that is isolated from the body by a semipermeable porous membrane. Membrane porosity can be tuned to prevent the entrance of immune cells and control the transport of molecules both into and out of the device.

pathogens found in the body [9,19,20]. Unfortunately, these rejection mechanisms also instruct the host immune system to recognize and respond to cells and materials, which have been implanted therapeutically. Thus there are various immune mechanisms, discussed later, that immunoisolation devices must overcome in order to protect therapeutic donor cells and materials from rejection by the host immune system.

Rejection pathways

Transplanted cell rejection may occur following direct cell contact between host and donor tissues, or through other, noncontact-initiated mechanisms (indirect activation) (Fig. 22.2) [9]. In direct rejection, cell−cell contact occurs between immune cells of the host immune system and the transplanted donor cells, which express as nonself cell surface markers [19,21−27]. This leads to direct activation of $CD4^+$ and $CD8^+$ T cells of the host immune system [26]. Once activated, the cytotoxic $CD8^+$ T cells initiate a cellular response against the donor cells, secreting soluble molecules which lyse the target cells and may initiate inflammation. In addition, $CD4^+$ T helper cells secrete a number of other factors (cytokines and chemokines) that stimulate other cells of the host immune system and help initiate the humoral response [28,29]. Activated macrophages may also recruit additional cell types and can secrete small molecules that can be toxic to donor cells. The activation of B cells leads to the generation of antibodies that can target donor cells. This forms part of the humoral response that also includes the activation of the complement and coagulation cascade [19,30,31].

The use of an appropriately sized immunoisolation barrier can prevent the initial cell−cell contact driving the direct rejection pathway; however, it may not offer protection against immune-activating factors that can diffuse across the barrier, as discussed in detail later. Here, antigens can be released from donor cells due to cell death, cell secretion, or cell shedding and may be small enough to cross the porous membrane protecting the transplanted cells [32−34]. These antigens can be processed by the host antigen presenting cells (APCs) and displayed on the surface of the APCs as part of the major histocompatibility complex [25,35]. Effector cells of the host immune system, such as $CD4^+$ cells, can then recognize the foreign antigen and activate the cellular and humoral immune response as described earlier [25]. In general, immunoisolation devices must provide a barrier to prevent foreign cell contact with the host immune system and prevent activation of the direct pathway. However, even without direct cell−cell contact, immunologic small molecules secreted by encapsulated cells may be able to activate the immune system [32−34]. The porosity of the immunoisolation barrier therefore plays an important role in controlling cell encapsulation and nutrition and may also impact the antigen load escaping from the device, discussed later.

Cellular nutrition

The cells inside immunoisolation devices require nutrients to produce and secrete therapeutic proteins. Nutrients include essential molecular building blocks, such as amino acids, nucleotides, lipid precursors, and molecules important for energy production such as glucose and oxygen. In addition, waste products must be removed, as the buildup of by-products from metabolism (lactic acid, carbon dioxide, and nitrogenous products) can negatively affect the viability of the cells [13].

The exchange of nutrients and waste products in a prototypical encapsulation device occurs by diffusion. Nutrients must diffuse from blood vessels into the interstitial fluid and then diffuse through the semipermeable device barrier to reach the cells in the core of the device. The reverse is true for waste products. The rate of diffusion or mass transport of nutrients can be impacted by a variety of factors. This includes the device architecture/geometrical features, porosity of the semipermeable barrier, nutritional reserves of the transplantation site, the density of cells within the device, and the specific metabolic requirements of the therapeutic cells being utilized.

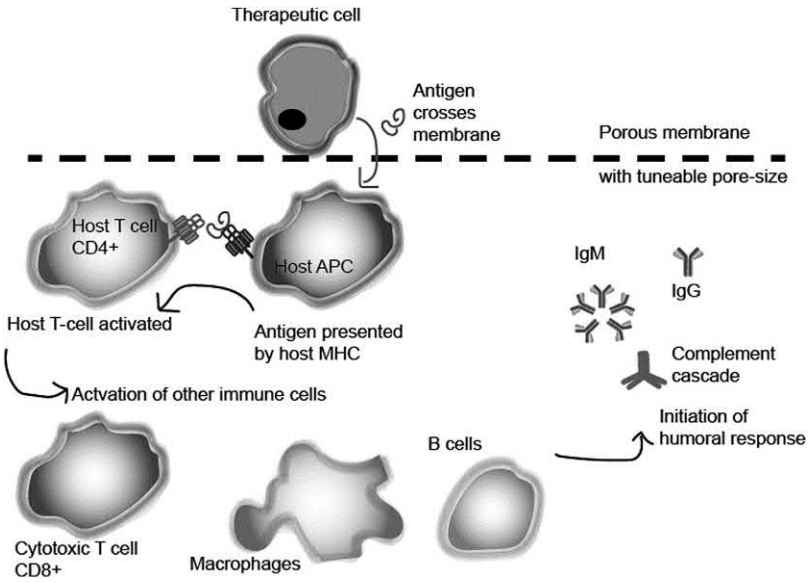

FIGURE 22.2 An overview of the indirect rejection pathway in which the host immune system can recognize foreign encapsulated cells without direct cell−cell contact.

Ideally, the barrier should be as porous as possible to allow for maximal diffusion of nutrition to the cells. However, this can also compromise the protective capacity of the device. Tuning the pore-size of materials utilized for immunoisolation devices can be used to optimize cell nutrition while preventing direct activation and host immune rejection. Mammalian cells are typically in the order of 5−15 μm in diameter. Many immunoisolation devices are therefore composed of cross-linked hydrogels or porous membranes with sizes smaller than this to ensure that host immune cells cannot penetrate into the device and initiate transplanted cell rejection through the direct pathway. However, certain immune cells and cancer cells are able to extravasate from blood vessels through relatively small gaps [36]. In fact, both T cells and macrophages have been shown to crawl or squeeze through small apertures on the order of micrometers and smaller than the typical diameter of a cell [19]. This suggests that pore sizes in immunoisolation barriers need to be in the submicrometer range to prevent host immune cell penetration and possible donor cell escape.

Assuming direct cell contact can be averted, there remain a number of immunological small molecules that may penetrate the immunoisolation barrier [27]. Alginate microcapsules have been used to deliver proteins [37,38], small molecules [39,40], and therapeutic antigens from encapsulated cells used as a vaccination strategy [34].

Donor antigens can be very small and include short peptide sequences [41,42]. These antigens are much smaller than the therapeutic proteins secreted by the cells (such as insulin ~6 kDa) and may be able to leak out of the immunoisolation device to initiate immune cell responses. In order to prevent donor cell destruction, efforts have concentrated on preventing the penetration or leakage of toxic and activating moieties both into and out of the immunoisolation device (Fig. 22.3). Therapeutic proteins such as insulin (6 kDa) [43] can be larger than immunogenic antigens that may be shed by the encapsulated cells [32−34]. The optimal membrane porosity may also be affected by the cell type encapsulated within the device. Allogeneic cells (same species: human into human) are likely to be less immunogenic than xenogeneic cells (cross species: pig into humans) and so may require less restrictive barriers than xenogeneic cells. While the immune system can become activated by these shed antigens [44], it is not clear whether the indirect pathway poses a significant block toward the translation of immunoisolation devices [45].

Therapeutic cells

A wide range of cells has been investigated for use within immunoisolation devices and can be categorized into three main types: primary cells, immortalized cell lines, and stem cells (Fig. 22.4).

Primary cells

Primary cells are isolated from human or animal donor tissues and include cadaveric islets or parathyroid tissue [13,14]. Primary cells are an attractive cell source as the cells are terminally differentiated and already contain the intracellular machinery required to secrete the therapeutic protein. They are typically nonproliferative and genetically stable. However, there are a limited number of human donor organs available, and these are insufficient to treat all patients. To overcome the limited donor supply, especially in the diabetes space, various strategies are

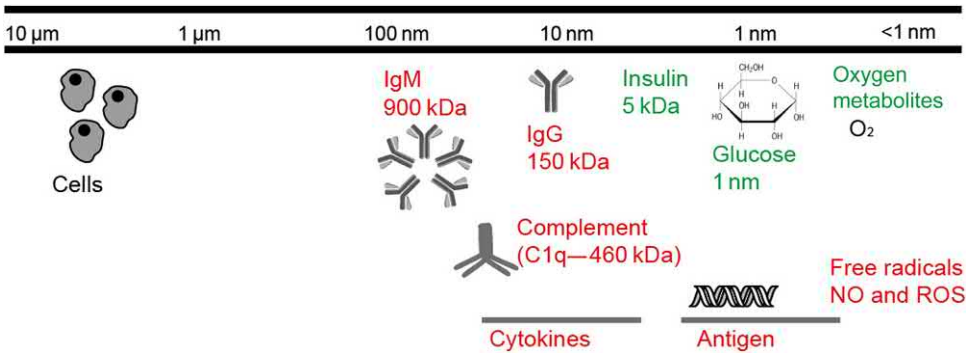

FIGURE 22.3 Immunoisolation barrier porosity as compared to biological length scales. Immunoisolation barriers must be able to transport essential nutrients and oxygen to encapsulated cells and allow for the transport of secreted proteins such as insulin. Some are designed to minimize transport of immunogenic or cytotoxic biomolecules (red).

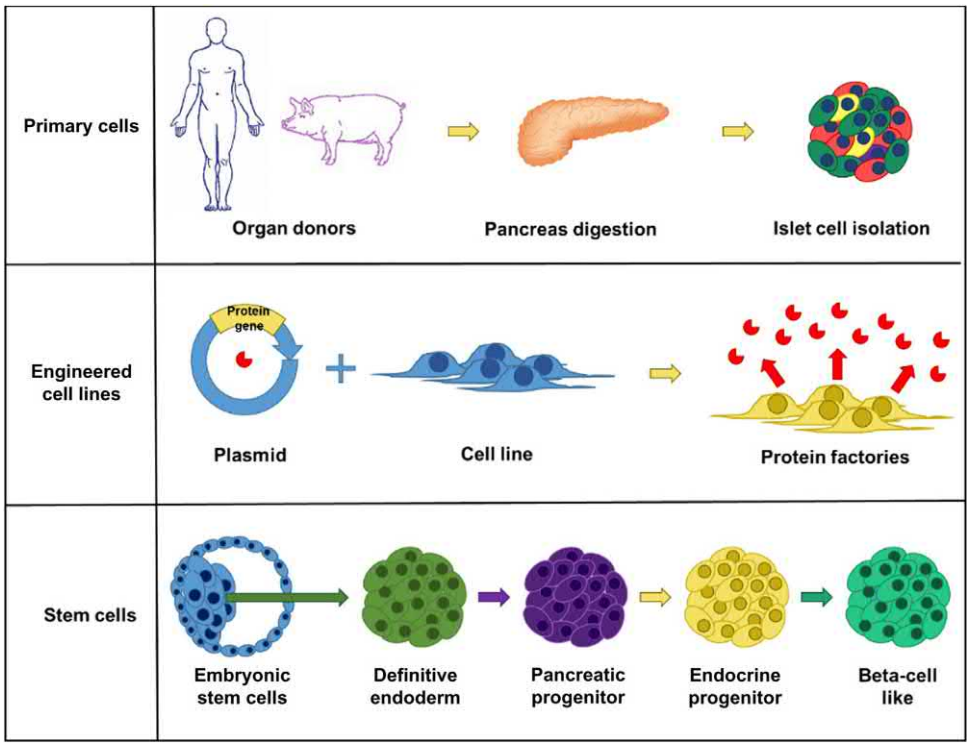

FIGURE 22.4 Cell types used in immunoisolation devices for cell-based therapies.

being developed to provide renewable islet cell sources. This includes porcine islets, proliferated beta cells to expand the number of islet cells isolated per donor, and insulin-producing cells differentiated from stem cells. The use of porcine islets as a renewable supply of differentiated islet cells is attractive but also has potential drawbacks. In particular, there have been concerns regarding cross species pathogen transmission of porcine endogenous retroviruses (PERV) and the production of specific glycans such as galactosyl-α-1 (Galα-1) by pig organs that are hyperimmune stimulatory in humans [46]. However, recent advances in genetic engineering of pig strains in Good Manufacturing Process (GMP) facilities have selectively targeted genes to make Galα-1 and other types of knockout pigs with safe PERV copy numbers to address these issues [47].

Immortalized cell lines

A wide range of immortalized cell lines can be engineered to secrete a therapeutic protein of interest. These can include murine mammalian cell lines that are transfected with agents to introduce genes necessary for producing recombinant proteins by the biopharmaceutical

industry such as Chinese hamster ovary cells, C_2C_{12} myoblasts, baby hamster kidney (BHK) fibroblasts, or mouse 3T3 fibroblasts [1,48]. However, similar to the description of Galα-1 above, there are posttranslational protein modification differences between rodents and humans. This can lead to the secretion of proteins by nonhuman cell sources that are recognized as foreign by the human immune system [26]. Human embryonic kidney cells (HEK293) and HT-1080 cells are two *human*-derived cell lines most often used in the biopharmaceutical industry for the production of protein therapeutics with potential in immunoisolation devices [1,49]. A cell line currently used in clinical trials for immunoisolation devices is human retinal pigment epithelial cells, which have been engineered to secrete ciliary neurotrophic factor to reduce macular degeneration [18].

Next-generation cell lines can be engineered using synthetic biology to secrete therapeutic proteins in a regulated fashion, often in response to known stimuli [50]. This engineered cellular control system allows cells to "turn on and off" protein production, which helps to limit the overproduction/secretion of excess proteins that may damage the body [51]. Synthetic biologists are inserting gene circuits that give cells the ability to sense a specific stimulus and then deliver a desired response [52]. Stimuli can include pH, mechanical forces, chemical signals, or specific energy waveforms, which then actuate a response—causing the cell to produce/secrete the desired protein [51,53–55]. For example, HEK293 cells were synthetically engineered to contain a gene circuit that can respond to applied external radio frequencies. When stimulated, this gene circuit activates the transgene for insulin production/secretion in vivo to help control diabetes [55]. By developing gene circuits with various input/stimuli genes and output/expression genes in a modular fashion, inputs and outputs can be simply interchanged. This facilitates modular genetic engineering of cells for a stimuli responsive protein secretion, depending on the respective disease [51].

Stem cells

Embryonic stem (ES) cells, inducible pluripotent stem (iPS) cells, mesenchymal stem cells (MSC), and other multipotent progenitors all have potential application as therapeutic cells within immunoisolation devices [56–60]. The ability to self-renew and then be differentiated into a specialized, quiescent state makes these cells attractive precursors to therapeutic cells. In addition, some MSCs have been shown to secrete various factors that reduce the immunogenicity of the device [61]. In terms of diabetes the use of stem cells to create an unlimited source of beta cells for widespread application of beta cell-replacement therapies has made significant progress [62]. Historically, stem cell generation of beta cells that contain all the complex intracellular machinery required for glucose-dependent insulin secretion (e.g., K^+ATP channels, voltage-dependent Ca^{2+} channels) has been difficult. But recent developments have elucidated the potential to robustly produce beta cell-like insulin-producing cells from stem cells in vitro. Following the developmental stages shown in Fig. 22.4, these beta cell-like cells have been shown to exhibit glucose-responsive insulin secretion in vitro and induce normoglycemia in diabetic rodent models posttransplantation, and multiple types of stem cells (ES, iPS cells from Type I diabetic patients, and pancreas progenitors) can be used to produce the cells [56–60]. Since undifferentiated ES cells have the potential to form teratomas or cysts, many groups are pursuing immunoisolation devices to protect both the cells and recipient from potential harm [63].

Device architecture and mass transport

The device shape and architecture govern the mass transport and exchange of molecules into and out of devices. The main shapes studied have been spherical, planar, and cylindrical fibers (Fig. 22.5). The shape then dictates the amount of surface area that contacts the interstitial tissue

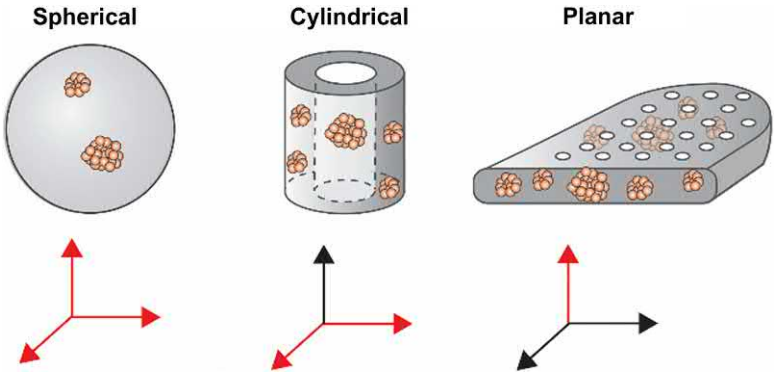

FIGURE 22.5 Main canonical design shapes and metabolite exchange directions (red).

for the exchange of molecules to occur with the body. Spherical designs use cells entrapped within microsphere hydrogels, and the porous hydrogel materials typically act as the immunoisolating barrier. Microspheres are attractive due to their high surface area-to-volume ratio and exchange in all x, y, z, directions. Cylindrical devices can consist of drawn hydrogels or hollow tubes with an outer immunoisolating membrane with exchange in x, y but not z directions. Planar designs contain a central cavity for the cells, which is sandwiched between immunoisolating membranes. Exchange can only occur in a maximum of two z directions.

Spherical devices are generally made from natural or synthetic hydrogels. Alginate is one of the most widely investigated cell microencapsulation materials [64]. This anionic polysaccharide derived from seaweed forms a hydrogel in the presence of divalent cations under cell-friendly conditions [64]. Briefly, islets are mixed with a viscous alginate solution and dripped into a divalent cation solution. This ionically crosslinks the alginate to solidify the gel, thereby entrapping islets within the alginate network [65]. Typically, few islets are encapsulated per sphere, which commonly range in diameters from 0.5 to 1 mm [66–69]. Several sphere formulations, with tunable ionic cross-linking and alginate composition, have demonstrated long-term diabetes correction in rodent models [45,66,70,71]. These promising results prompted the investigation of these alginate-based formulations in three small scale trials involving human patients. Unfortunately, these devices did not provide long-term glycemic control in patients [72–75]. One of the contributing factors to loss of cell function within alginate microspheres is a host fibrotic response to implanted materials. This causes collagen deposition and reduced oxygen and metabolite diffusion into the spheres [76]. Overcoming the host fibrotic immune response is a major challenge for the long-term survival of encapsulated cells that we discuss in a separate section later.

A number of cylindrical macrodevices have been developed to treat retinal degeneration and diabetes [18,77]. The TRAFFIC (thread-reinforced alginate fiber for islets encapsulation) device consists of an alginate fiber with dispersed islets that is mechanically reinforced by a central thread. The central thread uses two twisted nylon sutures that are coated with a poly(methyl methacrylate)/N,*N*-dimethylformamide/calcium chloride (PMMA/DMF/$CaCl_2$) solution to impregnate the central fiber with Ca^{2+} ions. The fiber is then placed into a cylindrical mold with an alginate/islet solution where the calcium ions slowly elute from the fiber to cross-link the alginate network. The TRAFFIC device was tested with rat islets in a diabetic C57bl/6 mouse model of diabetes and was reported to cure 10/14 mice for up to 3 months. The singular device could be retrieved from the mice, and all the cured mice returned to a hyperglycemic state indicating that the glycemic control was due to the TRAFFIC device [77]. This approach of using a reinforced hydrogel matrix could be used with a variety of hydrogel materials or could be impregnated with a range of other bioactive molecules for localized release of vascularizing agents and immunomodulatory drugs.

The shape of the immunoisolation device also controls the number of cells that can be implanted. Microspheres contain few cells per device; therefore multiple devices are required to be transplanted into a patient. Macrodevices, often planar or cylindrical, can contain many cells within one or a few devices. Each of these parameters (shape, size of device, and number of devices needed) presents advantages and disadvantages that must be tuned to the requirements of the specific disease being treated. For instance, microspheres may have beneficial diffusion properties for the nutrition of the therapeutic cells but may be more difficult to retrieve in cases where they need to be replaced or recovered. Macrodevices may be retrieved as a singular device but may have limited exchange kinetics that makes it difficult to support many cells within one device. These factors are interlinked with oxygen demands and the environment of the transplant site.

Transplantation site

Various transplantation sites have been investigated for the implantation of immunoisolation devices, and the appropriateness of the site must take into account specific requirements of the disease, device size and shape, and the cell type utilized. When choosing a transplantation site, careful consideration should be taken to determine the number of cells/protein required, volume of space to hold the device, metabolic requirements of the cells, the nutritional capacity of the site, immune cell activity of the site, transportation of drug from the site to the disease target, and the ease of access for transplantation [78]. These considerations can then guide the design of the device. Many transplantation sites have been investigated for devices, including the liver, kidney, bone marrow, intramuscular, subcutaneous, peritoneal cavity, pleural space, and epididymal fat pad [79–84]. The eye is an interesting space that has shown efficacy in clinical trials for macular degeneration using a very small implanted device [2,18]. This location is proximal to the disease site and is immune privileged: it is not readily infiltrated by immune cells that can cause device failure. A few key transplantation sites are elaborated next.

Subcutaneous (SC): The subcutaneous space offers ease of access through a minimally invasive implantation surgery. Devices implanted in SC spaces can be more easily retrieved in case the cells need to be replaced [85].

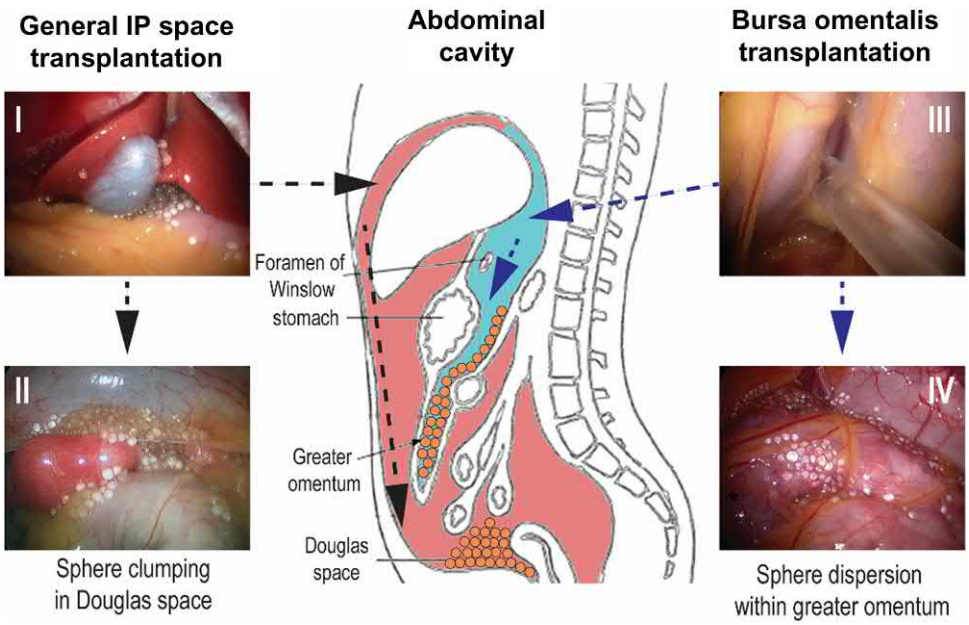

FIGURE 22.6 Schematic depicting the laparoscopic transplantation of immunoisolation devices into the intraperitoneal space and bursa omentalis. The settling of spherical devices due to gravitational sedimentation in bipedal primates was recently shown to be decreased in the bursa omentalis. *Adapted from Bochenek MA, Veiseh O, Vegas AJ, McGarrigle JJ, Qi M, Marchese E, et al. Alginate encapsulation as long-term immune protection of allogeneic pancreatic islet cells transplanted into the omental bursa of macaques. Nat Biomed Eng 2018;2(11):810–21.*

However, this site has also demonstrated suboptimal nutrition and low fluid turnover [86]. To improve the nutritional properties of the SC site for islet transplantation, various engineering strategies have been employed to encourage vasculature in the SC site and within devices, discussed later.

Intraperitoneal space: The intraperitoneal space within the abdominal cavity offers a large volume of space to hold multiple devices (Fig. 22.6). The site can be accessed via a noninvasive laparoscopic procedure, has high fluid turnover from the circulation of peritoneal fluid, and the omental tissue is vascularized but concerns have been raised regarding oxygen availability for implanted cells [3,87,88].

Bursa omentalis: Also located within the abdominal cavity, the bursa omentalis or lesser sac, is formed by a tissue bilayer of the greater omentum (Fig. 22.6). The sac hangs like an apron on top of the intestines and below the intraperitoneal space. The tissue bilayer of the bursa omentalis was recently shown to reduce the settling and clumping of implanted spherical devices that occur in bipedal primates due to gravity sedimentation [3]. Any clumping of immunoisolation devices can lead to enhanced fibrotic responses driven by the host immune system and reduce nutrition for any cells housed in the center of the clumps.

Improving oxygenation of immunoprotected cells

Various strategies have been employed to improve diffusion kinetics and oxygenation of immunoisolation devices (Fig. 22.7). These include maximizing the porosity of the device using superthin porous immunoisolation barriers to reduce diffusion distances [89]. Alternatively, oxygenation can be enhanced by increasing vasculature around the device, including oxygen carriers to enhance oxygen transfer, in situ oxygen generation, an exogenous source of oxygen, or reclustering cells into smaller cell aggregates [79]. Some cell aggregates used for immunoisolation devices are too large for oxygen to adequately reach cells in the center. This can lead to cell death and central necrosis in the core. After dissociation and reaggregation into small aggregates, cells can obtain more oxygen and have been shown to function better under hypoxic conditions and secrete lower levels of proinflammatory biomolecules such as MCP-1 or IL-6 [90].

Many groups have investigated methods to increase vasculature around immunoisolation devices [91–93]. In one approach, researchers induce neovascularization in the transplant site by implantation of a silicone catheter weeks prior to implantation of islet cells. This was reported to improve islet functionality by reducing hypoxia-related islet cell death in the peri–post transplantation period [86]. The delivery and scaffold release of vascularizing ligands and soluble factors such as vascular endothelial growth factor (VEGF)/PEDGF have also been employed to generate neovasculature at the transplant site [1]. One of the first planar devices, TheraCyte, consisted of a hollow chamber surrounded by an immunoisolating membrane. This was then covered with a second membrane that was designed to induce neovascularization for nutrition of the cells at the transplant site [94]. Early studies demonstrated the ability to pretransplant the device to

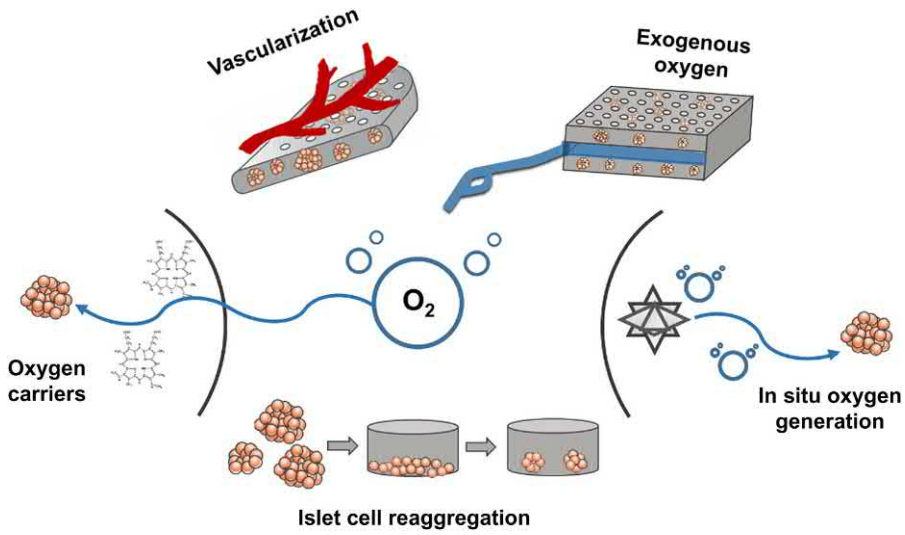

FIGURE 22.7 Strategies to improve oxygenation and enhance cell nutrition.

induce neovascularization in rats prior to loading the allogenic islet cells through a port [95,96]. Currently, studies are being completed with a next-generation device (VC-01) that utilizes a similar macrodevice strategy. VC-01 was reported to support the differentiation of human pancreatic progenitor cells into mature beta-cell like cells posttransplantation using an in vivo mouse model [43]. These results led to a Phase I clinical trial where human stem cells, CyT49, were partially differentiated into pancreatic progenitors (PEC-01) and loaded into the VC-01 devices to investigate the potential for these cells to form into human beta-cell like cells. Unfortunately, of the 19 patients transplanted with devices, only the devices retrieved from two patients were found to contain live cells, while the majority contained few viable cells [63]. The failure of these devices to support cell viability was largely attributed to the foreign body response to the vascularizing membrane [63].

Efforts have also focused on alternative methods of increasing oxygenation synthetically. Perfluorinated compounds or inclusion of hemoglobin substitutes within immunoisolation materials have been investigated as oxygen carriers to enhance the diffusivity of oxygen [97–99]. These have been reported to improve islet cell responses under hypoxic conditions [99]. The OxySite device encapsulates CaO_2 crystals within polydimethylsiloxane (PDMS) disks to provide in situ generation of oxygen for therapeutic cells. PDMS is hydrophobic and slows the hydration of CaO_2 crystals; incorporation of CaO_2 crystals in hydrophilic materials was shown to release oxygen too quickly and induced hyperoxia, which reduced cell viability [100].

The Beta-O_2 device βAir consists of a planar disk with a refillable oxygen chamber surrounded by an immunoisolating membrane. [101] Islets are encapsulated in alginate slabs and placed on each side of the central gas chamber. Ports are connected to the central gas cavity and allow for the daily refueling of oxygen by injection through the subcutaneous port. The whole device is then wrapped in a polytetrafluoroethylene (PTFE) immunoisolating membrane and is transplanted in a subcutaneous pocket [102]. This device has shown efficacy in various diabetic animal models including rats [103], minipigs [104], and nonhuman primates (NHPs) [105], using both allogeneic and xenogeneic islets cell sources. The βAir device is currently in clinical trials, and the first five human patients have been transplanted with a subtherapeutic islet dose [101,106]. The islets have been shown to remain viable and functional in the device for up to 2 years in patients but have only shown moderate improvements in the clinical disease state. The patients were transplanted with a low number of islets (2,100−4,600 islet equivalence (IEQ)/kg patient), which is less than the 10,000 IEQ/kg recommended dose for intraportal islet transplantation [6]. The investigators reported no signs of human leukocyte antigen (HLA) immunization to the foreign islets within the devices. However, a substantial foreign body response and fibrotic capsule around the device was reported in three of four patients in the most recent trial. Increasing the number of islets per device and reducing the thickness of the fibrotic capsule may be ways of improving the kinetics of insulin secretion out of the device in the future.

Controlling immune responses to implanted materials

Immunoisolation devices are designed to protect transplanted cells from the rejection by the host immune

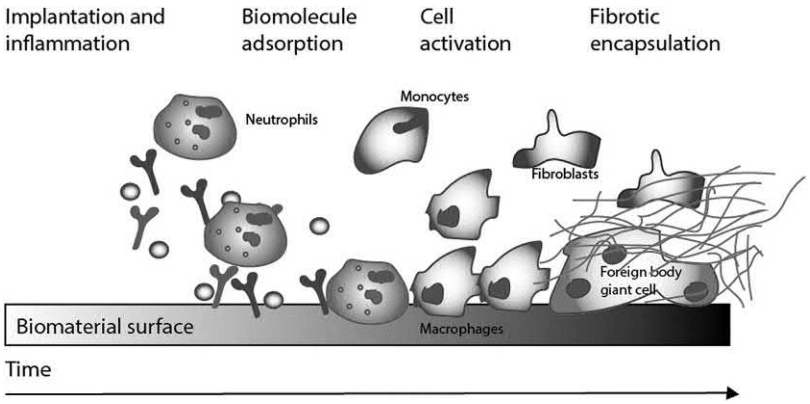

FIGURE 22.8 An overview of the steps in the foreign body reaction. Postimplantation inflammation leads to adsorption of proteins and biomolecules onto the biomaterial surface and the influx of neutrophils. Monocytes and macrophages are activated, causing macrophage fusion into foreign body giant cells. The recruitment of fibroblasts leads to deposition of a collagen matrix and fibrotic encapsulation.

system. However, the immune system may also recognize and react to these implanted materials themselves. Implanted materials can cause the immune system to initiate a foreign body reaction (FBR), which can eventually lead to collagen deposition around the implant [107,108]. This deposition of collagen can form a fibrous capsule around implanted materials, which impairs device function. In the case of transplanted therapeutic cells, it can leave cells deprived of oxygen and at risk of necrosis. Thus a successful immunoisolation device must both protect the transplanted cells from host immune rejection and minimize the FBR to the device. Here, we discuss the FBR and engineering approaches which can help to prevent immunoisolation devices themselves from provoking an undesired immune response.

Steps in the foreign body reaction

In general, the immune system identifies implanted materials as nonself and initiates the FBR (Fig. 22.8). Briefly, implanted biomaterials are exposed to blood and tissue fluids, which causes proteins to adsorb onto the biomaterial surface. These can then be identified by immune cells, which trigger acute (1−2 weeks) and then chronic (2 weeks +) inflammation, culminating in the fibrous capsule surrounding the device [109−111].

Several cell types are known to be involved in the foreign body response, which is mainly orchestrated by innate immune system cells, including leukocytes such as neutrophils, monocytes, and macrophages. Importantly, the recruitment and differentiation of monocytes/macrophages is a key step in the FBR [107,108,112−114]. First, in a process known as "frustrated phagocytosis," the macrophages try to engulf the large device and eventually fuse together to form large polynuclear foreign body giant cells [109,115,116]. These, along with other macrophages that have become activated surrounding the implant, secrete a cascade of inflammatory chemical signals to recruit other cells. These can include fibroblasts that then deposit collagen around the biomaterial, and ultimately, the fibrous capsule containing extracellular matrix proteins and immune cells [113,114,117,118].

The formation of this dense collagen layer and fibrotic capsule isolates the implant material from the rest of the body [113,119−121]. Unfortunately, this also isolates the implant from the local vasculature, often causing cells within the device to experience hypoxia and cell death. In order to combat FBR, material properties must be carefully selected or engineered to limit the key steps in the FBR process, including protein adsorption, inflammation, and immune cell recruitment/activation.

There are additional factors that can influence the foreign body response to implanted materials, including the biological site of implantation, species, and strain of host animal receiving an implant and donor cells, and the surgical technique used to implant the device. Furthermore, intrinsic material properties such as chemical composition, geometry, and morphology have also been shown to affect how the body responds to implants [122]. Engineers can tune immunoisolation devices to resist the FBR by targeting several stages, including protein adsorption, inflammation, and immune cell recruitment [3,123−128].

The role of geometry in the foreign body reaction

The size, shape, mechanical properties, and topography of implanted devices are emerging as factors that can affect the FBR. Many immune cells have been shown to be sensitive to the mechanical properties and underlying topography of their surroundings [129]. Materials with complex topographical features typically present increased surface area for protein adsorption and cellular attachment; however, topographical cues can affect cell behavior [130].

Several cell types, in particular human fibroblasts [131−136], can sense and respond to depressed and elevated nanotopographical features such as wells, pits, and

nanopillars. By altering the nanotopography of materials, cells can be directed to orientate around specific features or to reduce adhesion [131,136–139]. This strategy has been employed in osteo-implants, where changing surface roughness has been shown to impact cell adhesion and clinical outcomes [140]. The role of device geometry in controlling FBR has only recently been explored. For instance, it was found that by increasing the size of spherical devices from 0.5 to 1.5 mm diameter, the foreign body response could be reduced and was similarly observed across many sphere materials [141].

Many of these physical properties (size, shape, geometry, mechanical properties) are intrinsically linked, and the precise role of each of these components in directing cellular behavior in the FBR response has yet to be elucidated. Importantly, macrophages are a key regulator of the FBR, and it has been shown that macrophage phenotype can be modulated toward pro- or anti-inflammatory phenotypes by these properties [142–150].

Tuning chemical composition to prevent attachment

The chemical composition of the material can affect the FBR process. Chemical charge, hydrophobicity, and whether the material is derived from natural or synthetic sources can all impact the response to implanted materials at different stages in the FBR cascade. For example, coagulation Factor II (a component of the coagulation cascade) has been reported to bind to alginate–cellulose materials but not to ionically cross-linked alginate. When these materials were incubated in model systems, materials that could bind Factor II showed increased coagulation, which is an important precursor to downstream fibrosis [151].

Using this principle, immunoisolation devices that resist protein adsorption, or even cellular attachment, have been designed. By coating devices with hydrophilic molecules such as poly(ethylene glycol) or zwitterionic-based materials, both protein adsorption and cellular attachment can be reduced. This strategy of preventing the FBR through the early stages of protein absorption, or the later stage of cellular attachment, has proven useful in minimizing the fibrotic response to implanted devices [152–154]. Recently, a chemically modified microsphere formulation has also demonstrated the potential to reduce fibrosis of alginate-based formulations in mouse and NHP models. Alginate modified with a small-molecule was found to reduce macrophage adhesion and could protect viable and glucose-responsive islets in an NHP model for up to 4 months without fibrosis [3]. These systems were also shown to protect stem cell–derived beta cells and show long-term normoglycemia in immune competent mice with a renewable islet cell source [69,127].

Directing immune cell behavior in the transplant niche

One strategy to minimize the fibrotic response to an immunoisolation device is to deliver signals to the cells in the transplant niche to directly alter their behavior. Such approaches typically focus on suppressing unwanted immune cell responses and recruiting cells that may promote a more receptive transplant environment. For example, macrophages can display pro- or anti-inflammatory phenotypes. Suppressing proinflammatory macrophage activity through the delivery of soluble drugs has been shown to dramatically reduce the foreign body response to implanted materials [107,108,129,135,155–162]. Materials have been engineered to release a soluble form of the CSFR1 inhibitor of macrophage activation or the local delivery of the immunomodulatory protein chemokine CXCL12 and have been reported to decrease fibrosis [69,108,163]. When islets were encapsulated in microspheres, which also eluted these biomolecules, they were reported to induce long-term normoglycemia in diabetic C57bl/6 mice using a variety of islet sources (human, rat, and porcine).

Another microsphere design utilizes a combined approach; a double-coated sphere and the short-term administration of soluble immunosuppressive drugs CTLA4-Ig and/or anti-CD154 mAb for dual costimulatory blockade [164]. The inner core contains encapsulated islets, which is then coated with a poly-L-lysine (PLL) coating to provide a perm-selective immunoisolating membrane. The outer shell is composed of alginate to mask the PLL coating, which has been shown to be immunogenic [165,166]. Recently, various regimens of costimulatory blockade immunosuppression for transplant of encapsulated adult porcine islets were investigated in diabetic NHPs [68]. Results indicate that the immunoisolated islets could remain viable without fibrosis but demonstrated transient improvement in glycemic control of the diabetic NHP. Interestingly, the encapsulated islets could be retrieved from the NHP with transient glycemic control, and following transplantation into diabetic immune compromised mice, the retrieved encapsulated islets were now able to induce normoglycemia in the mice. This study suggests that there may be additional differences between the intraperitoneal transplant sites in mice and NHP, and differences in curative transplant demand due to oxygen or insulin requirements in different host species.

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Part Six

Stem cells

Chapter 23

Embryonic stem cells

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Introduction

Tissue engineering relies on the availability of cells capable of repairing or replacing damaged tissues and organs. Although multiple sources of cells from cadavers to animal tissues have been considered, pluripotent stem cells (PSCs) are of intense interest because they can be expanded indefinitely in culture and can also be directed to differentiate into a particular cell type of interest. Adult tissue-derived stem cells such as those from bone marrow, fat, and skin are among the most studied and while some are currently being used in the clinic, these sources are donor-dependent, have limited expansion potential, and a highly restricted differentiation capacity. Ideally, having a single inexhaustible source of pluripotent cells that can generate large quantities of therapeutic cells would greatly facilitate regenerative medicine endeavors while simplifying quality control and safety testing. Human embryonic stem cells (hESCs) offer the unique possibility of meeting these criteria and thus may be able to circumvent many of the problems associated with variations in donor-dependent cell sources.

Derivatives of hESC have been regarded by many as a panacea for the treatment of various ailments, ranging from osteoarthritis to blindness, heart disease, and spinal cord injuries, among others. The list is long and numerous promising studies in animal models indicate that the question now is not whether hESC-based therapies could be safe and effective but what indications are they best suited for and how soon can they become a standard of care. Before this happens, however, several unique challenges need to be overcome. For example, the generation of hESCs is wrought with controversy as the standard derivation process involves destruction of a developing human embryo. Similar to adult tissue-derived cell sources, another issue that needs to be addressed concerns histoincompatibility between hESC derivatives and the intended recipient. The advent of induced PSCs (iPSCs) could one day alleviate both the ethical and histoincompatibility issues surrounding the use of hESC [1,2]. iPSC can be generated from a person's own mature somatic cells and through the process of reprograming, turned back into a pluripotent state for use as a patient-specific renewable cell source with broad differentiation capabilities. Histoincompatibility may be managed through a variety of different approaches, including the use of immunosuppressive drugs, the generation of hESC banks to help match human leukocyte antigen (HLA) haplotypes between the starting hESCs and the intended recipient, and genetic engineering to make hESCs or allogeneically used iPSCs (and their derivatives) universally immunocompatible. This chapter will focus on hESC derivation and progress toward clinical application of their derivatives yet much of the knowledge that has been gained through this research is applicable or even transferable to the iPSC field.

Approaches to human embryonic stem cell derivation

hESCs were first isolated in 1998 from the inner cell mass (ICM) of a preimplantation human blastocyst in a landmark study by Thomson et al. [3]. As research with hESC gained interest, great promise as well as great controversy was brewing in the press. hESC theoretically have the capability of giving rise to all tissues of the human body and may provide crucial therapeutic treatment for a wide variety of diseases yet the only known hESC derivation process back in the late 1990s/early 2000s involved destruction of a human embryo, and this was seen by some as the equivalent of killing a human being. Acknowledging the enormous potential of hESC research while also trying to quell the ethical firestorm that hESC derivation had garnered, in 2001, the Bush administration made the decision to allow federal funding

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for hESC research on the condition that only hESC lines already in existence could be used. The NIH hESC registry list (http://stemcells.nih.gov/research/registry/) was established to keep track of approved lines. The original hESC lines derived by Thomson et al. at the University of Wisconsin [3] were among the first hESC lines registered and have become the most widely used and published hESC lines available. Since then, however, many other hESC lines have been derived using private funds and other stem cell banks and registries have been created. Examples include the University of Massachusetts International Stem Cell Registry [4], European Human Embryonic Stem Cell Registry [5], and UK Stem Cell Bank [6].

hESCs retain a fundamental property of ICM cells—the ability to give rise to all tissues of the human body. The same markers of pluripotency are found in both ICM and hESC—the transcription factors Oct-4, NANOG, Rex-1; cell surface antigens SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (Fig. 23.1); and high alkaline phosphatase activity [7,8]. Cells of the ICM only exist for a few days in the natural course of embryo development; they arise during blastocyst formation from a morula on day 4 and persist throughout the first days or hours postimplantation before beginning the highly orchestrated process of differentiation. ESCs may be seen as an immortal extension of the ICM in culture; they retain high telomerase activity and can continue to self-renew indefinitely under appropriate culture conditions. When injected into immunodeficient mice, hESCs form teratomas—tumors that contain derivatives of all three germ layers, the most typical ones being bone, cartilage, neural rosettes, and epithelium of the airways and gut. Like the ICM of a developing blastocyst, pluripotent ESCs in culture are primed to differentiate, and as we will discuss later in the chapter, this makes working with them both easy and difficult at the same time.

Traditionally, ESCs have been derived from day 5−7 blastocysts with or without immunosurgery (removal of the trophoblast) by plating a blastocyst or an isolated ICM (Fig. 23.2) on a feeder layer of mitotically inactivated mouse embryonic fibroblasts [3,9]. Yet, new lines have now been derived using alternative approaches in an attempt to overcome ethical issues surrounding embryo destruction during hESC derivation [10,11]. Moreover, the use of mouse feeder cells and animal products during hESC derivation and maintenance has been eliminated in newer methods because of safety concerns that they could harbor unknown xeno viruses. Producing cells that are compatible with a patient's own immune system is also an important consideration for preventing rejection. Thus at present, hESCs have been derived on human feeders [12−14] and even on feeder-free matrices [15]; they have

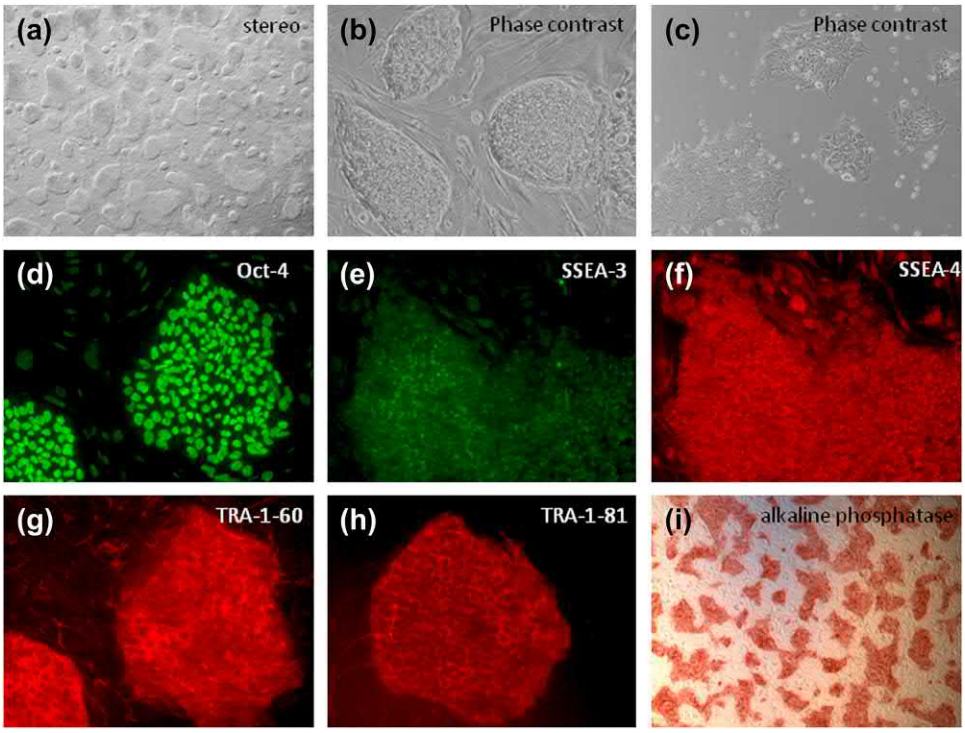

FIGURE 23.1 Morphology and markers of hESC. (A, B)—hESC grown on feeder cells, (C)—hESC grown on Matrigel, (D−I)—markers of pluripotency in hESC. (A)—Stereomicroscope image (as seen during mechanical dissection), (B, C)—phase contrast, (D−H) immunofluorescence, (I)—bright field. (D)—Oct-4, (E)—SSEA-3, (F)—SSEA-4, (G)—TRA-1-60, (H)—TRA-1-81, (I)—alkaline phosphatase. Magnification: (A) ×10, (B, D−H) ×200, (C) ×100, (I) ×4. *hESC*, Human embryonic stem cell.

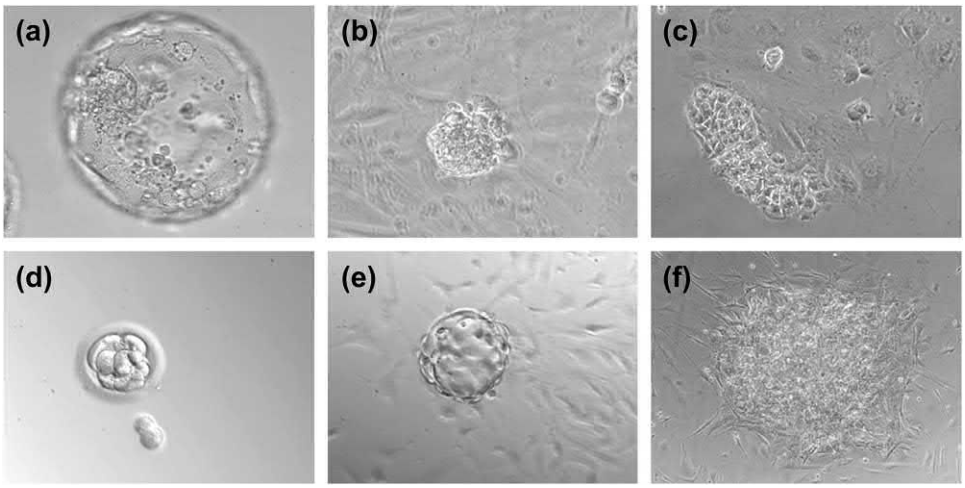

FIGURE 23.2 Early stages of hESC derivation from a blastocyst (A–C) and a single blastomere (D–F). (A)—Expanded blastocyst, (B)—ICM after immunosurgery plated on feeder cells, (C)—ICM outgrowth, (D)—biopsied embryo and a developing blastomere, (E)—developing blastomere before plating on feeder cells, (F)—blastomere outgrowth. *hSEC*, Human embryonic stem cell; *ICM*, inner cell mass.

been derived from growth-arrested in vitro fertilization (IVF) embryos that cannot be used for implantation [16], from unfertilized oocytes that are incapable of forming a developmentally competent embryo [17–20], and from a single blastomere of a morula stage embryo—a technology that allows the generation of hESC while leaving the embryo unharmed [14,21–23].

Derivation of hESC from chemically activated unfertilized oocytes [parthenote hESC (hPESC)] was reported by several groups [17–20]. These studies showed that hPESCs are very similar to ICM-derived hESCs: they have the same colony morphology and growth behavior, maintain a normal karyotype, express the same markers of pluripotency, and differentiate into derivatives of all three germ layers. These cells could also potentially address the ethical and immune compatibility issues that plague traditional hESC lines, as parthenogenetic hESC lines from carefully selected donors could match a larger proportion of the population because they have a reduced number of HLA. However, some studies showed that a large percentage of parthenogenetic blastomeres were affected by an excessive number of centrioles, a high aneuploidy rate [24], as well as genetic and epigenetic instability [22]. A lot of progress has been made generating hPESC and their derivatives [25–31]. Although the technology is not very widely used, one group has differentiated their clinical-grade hPESC line into midbrain dopaminergic (DA) neurons and begun testing it in Phase 1/2a clinical trials to treat Parkinson's disease (PD) (www.clinicaltrials.gov, clinical trial ID# NCT03119636). Preclinical studies of these hPESC-DA neurons in a non-human primate model suggested that they are safe to use and provided functional improvement in behavioral endpoints for up to 24 months [32]. It remains to be determined if hPESCs can compete with "conventional" hESCs for the generation of cells for regenerative medicine.

A technically challenging yet innovative approach to hESC derivation is based on somatic cell nuclear transfer (SCNT). During a micromanipulation procedure, the nucleus of a donor cell is introduced via a micropipette into the cytoplasm of an enucleated unfertilized egg, which then develops into a blastocyst. ESC isolated from such blastocysts would have the same genotype as the donor of the nucleus, thus their derivatives could be used as autologous transplants. SCNT can also be performed by fusing a donor cell to an enucleated oocyte. Successful generation of SCNT-hESC was reported by several groups who used fetal, neonatal, and adult fibroblasts as donor cells for somatic nuclei [33–35] with a success rate of up to 25% [36]—efficiency comparable to that of hESC derivation from naturally fertilized blastocysts. The efficiency of blastocyst formation and hESC derivation though varied for eggs obtained from different donors and could even correlate with the hormonal stimulation protocol [35]. Another major SCNT reprograming barrier was shown to be associated with severe methylation of lysine 9 in histone H3 in a human somatic cell genome [36], but introduction of KDM4A, a H3K9me3 demethylase during SCNT, significantly improved the development of SCNT embryos. Interestingly, this approach worked even when researchers deliberately used the eggs from the same donors whose eggs previously failed to produce SCNT blastocysts. While the possibility of creating donor-matched hESC lines by SCNT remains attractive, successful implementation of this technique takes years for the operator to develop skills required to perform this procedure with high precision and efficacy and minimal disturbance to the egg or donor nucleus. One of the groups that have been able to successfully derive SCNT-hESCs is now testing the safety and tolerability of their SCNT hESC–derived retinal pigment epithelium (RPE) in patients with advanced dry age-related macular degeneration (AMD) (NCT03305029). Yet, it is unlikely that

SCNT will be commonly used in the future to create new hESCs for clinical use given the many technical challenges that micromanipulation of nuclei presents and the difficulties in procuring suitable oocytes.

Another approach that has proven to be successful for generating hESC without embryo destruction is the use of embryo biopsy. This procedure, once commonly used in preimplantation genetic diagnostics in the course of IVF, involves removal of a single blastomere from a morula stage embryo [37] and has resulted in hundreds of healthy babies being born. Using this procedure, we established several hESC lines from single blastomeres while the donor embryos were allowed to develop to the blastocyst stage and cryopreserved [23]. Single blastomeres were first cocultured with the biopsied embryos and then plated on feeder cells in microdrops [21–23], and outgrowing colonies were treated the same way as ICM outgrowths (Fig. 23.2). In early experiments, blastomere outgrowths were cocultured with green fluorescent protein (GFP)-expressing hESC, which presumably helped condition the microenvironment and boost the outgrowth of hESC from blastomeres. After initial outgrowth was observed and the colony of blastomere-derived cells became large enough for passaging, GFP-negative colonies were marked under the fluorescence microscope and then mechanically dissected and replated; this procedure was repeated for several passages to ensure that the new cell lines were free of any contaminating GFP-expressing hESC. In addition, after the blastomere-derived hESC lines were established, they were extensively tested for the absence of GFP-positive hESC used for coculture and shown to be free of any GFP-expressing hESC [21,22]. Two hESC lines were generated that had similar properties to ICM-derived hESC: they stained positively for Oct-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, alkaline phosphatase and had a normal karyotype and differentiated into derivatives of all three germ layers both in teratoma assays in immunodeficient non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice and in vitro. In the first proof of principle study, the derivation yield was low, and the embryos were not preserved after the biopsy, but later this study was repeated using an optimized approach, which allowed the derivation of new lines from single blastomeres with higher efficiency and still without embryo destruction. Biopsies that were performed to isolate a single blastomere did not significantly affect the embryos; they developed to the blastocyst stage and were then frozen as expanded and/or hatched blastocysts [23]. Five hESC lines were produced this way from biopsied embryos and all of the embryos developed into healthy-looking blastocysts prior to cryopreservation [23]. This technique was subsequently used by several other groups to derive hESC lines with high efficiency and/or without destroying the embryos under different conditions including human feeder cells, feeder-free, and even from growth-arrested embryos [14,38–43]. Single blastomere–derived hESC showed a similar transcriptional profile to "conventional" hESC [44], and several differentiation derivatives of single blastomere–derived hESC appeared functional in vitro and in vivo in preclinical studies [45–49]. Single blastomere–derived hESC lines have already proven to be a useful starting material for regenerative medicine—one such line, MA09, was among the first of any type of hESC to be used clinically. The safety and tolerability of MA09-derived RPE was demonstrated in Phase 1/2 clinical trials in patients with AMD and Stargardt's disease, the juvenile form of AMD [47,48,50]. By avoiding destruction of human embryos, this technique also addresses the ethical concerns surrounding hESC derivation thereby offering a way to overcome a major hurdle in developing hESC-based therapies.

An interesting new development in hESC generation is the so-called naïve hESC. The term comes from the research on mouse ESCs, which have been shown to exist in two states, "naïve" and "primed" [51–54]. In addition to expressing markers of pluripotency, such as Oct-4, NANOG, and Sox2, pluripotent cells have both X-chromosomes active. Primed ESCs, coming from the epiblast, or EpiSCs, retain the expression of these markers, but only one X-chromosome is active [55,56]. hESC resembles mouse primed ESC rather than truly naïve cells. There are differences in morphology: the forms of hESC colonies are flat, not dome-like as typical for naïve cells, and they are polarized [57]. Metabolism of pluripotent cells relies on glycolysis, while primed cells use oxidative phosphorylation [58,59]. In primed ESC, only one X-chromosome is active, and they are very sensitive to single cell dissociation [51,60]. RNAseq analysis of single cells of human blastocysts showed that at least three types of cells can be identified and that during derivation of hESC, there are changes in gene expression as the cells are adapting to culture conditions [61], but it is still unclear if primed cells already exist in the embryo, or the transition happens during the first steps of hESC derivation.

Mouse ESCs are usually derived and exist in a naïve state, and it takes a special effort and culture conditions to isolate primed mouse ESCs (EpiSCs) from an epiblast. On the opposite, most hESC lines were derived and propagated in a primed state, until naïve, or "ground state" pluripotent hESC were derived [62,63]. Naïve hESC can be established de novo or through conversion of existing hESC lines to the naïve state;[64,65]. In addition, "naïve" iPSC can be established through reprograming under specific culture conditions [66]. In these cases, signaling pathways need to be activated or inhibited using bioactive substances and small molecules, such as the MEK inhibitor PD0325901, GSK inhibitor CHIR99021, STAT3

inhibitor NSC74859, ROCK inhibitor Y27632, LIF, bFGF, and TGF beta 1.

Once established, naïve cells survive single cell dissociation much better, which allows for greater expansion capacity (which could be highly desirable for large-scale manufacturing), their doubling time is much shorter, and they have been shown to differentiate more efficiently both in vivo and in vitro [67,68]—a highly sought after property when differentiation is aimed at making derivatives for regenerative medicine.

Maintenance of human embryonic stem cell

In vivo, the pluripotent cells of the ICM exist only transiently, differentiating into three lineages within a few days after the formation of a blastocyst. Maintaining hESC, the extension of ICM cells, in vitro has been a challenge due to their intrinsic propensity to differentiate as soon as the colonies reach a certain "critical mass." Thus hESC culture conditions have been optimized to prevent such spontaneous differentiation of hESC and maintain them in a pluripotent state over an infinite number of passages. Currently, there are several popular hESC culture systems and with a little optimization, all of them can be used to grow pluripotent cells with relative ease. Mouse embryonic fibroblasts or human fibroblasts derived from fetuses, placenta, or foreskin have been used as feeders for many years, and the corresponding medium for this culture system is based on Knockout-DMEM (KO-DMEM) and Knockout Serum Replacement (KSR, both components from Invitrogen), supplemented with at least 8 ng/mL human bFGF [3,8,12,21–23]. Variations of this system include addition of Plasmanate (plasma protein fraction from pooled donor plasma) [8–10,19] or using a 1:1 mix of KO-DMEM and F12 medium, or feeder-produced extracellular matrix instead of live feeder cells. On feeders, cultured hESCs have a very unique morphology and are best described as well-defined colonies of small tightly packed epithelial-looking cells with a high ratio of nuclei to cytoplasm and visible nucleoli (Fig. 23.1). When grown in KO-DMEM:F12 media mix or in the absence of feeders (i.e., "feeder-free"), hESC can deviate from this phenotype and look more spread-out and even grow as a sheet of cells; however, such cells still retain their pluripotency and are able to both self-renew and differentiate (Fig. 23.1).

A robust and user-friendly feeder-free system that employs a very dilute Matrigel solution (extracellular matrix produced by Engelbreth-Holm-Swarm mouse sarcoma cells), and matching defined medium was introduced by Stem Cell Technologies. Another feeder-free system, made by Invitrogen utilizes CellStart, a human placenta–derived extracellular matrix. Feeder-free protocols are usually provided by individual manufacturers together with a recommended medium and should be optimized by the researcher. In our own studies, we used extracellular matrix produced by mouse embryo fibroblasts, which was deposited and assembled by live cells and provided a three-dimensional growth surface similar to that provided by live feeder cells. This matrix was used in combination with KSR/Plasmanate-based culture medium and was successfully used for derivation of hESC. Derivation of an hESC line when the initial outgrowth consists of only a few cells is more challenging than routine culture of millions of cells, and this culture system proved to be very supportive of such a demanding task [15].

Avoiding animal-derived components and especially, live animal feeder cells, in derivation and maintenance of hESC is desirable for regenerative medicine applications, as this makes the derivatives of hESC more "clinic-friendly." Currently, there are several media–matrix systems on the market that are free of animal-derived materials. Such include Nutristem (Biological Industries, Israel), TeSR2 (Stem Cell Technologies, Canada), and StemFit (Ajinomoto, Japan) media, xeno-free laminin (Biolamina, Sweeden), iMatrix (Nippi, Japan), Vitronectin (Stem Cell Technologies, Canada)—and the number of such materials is growing.

Subculture of human embryonic stem cell

ICM of a developing blastocyst follows the differentiation program after implantation giving rise to derivatives of three germ layers. Pluripotent ESC, and in vitro "extension" of the ICM, can readily and rapidly differentiate when the microenvironment changes. This propensity of hESC makes them a unique source of differentiated cells for regenerative medicine, but at the same time it also creates many maintenance challenges. Spontaneous differentiation can happen regardless of most carefully chosen media–matrix combinations as a result of the colonies reaching a certain size, so the cells inside the colony have special limitations for self-renewal and commit to differentiation. Timely passaging disrupts differentiation-inducing signaling coming from complex cell–cell and cell–matrix interactions established during the first few days at each passage, so newly plated single cells and small cell clumps remain in pluripotent stage and continue to self-renew.

To subculture hESC, several methods can be employed. Mechanical colony dispersion and hand-picking has been the gold standard in the field as it allows one to select colonies of correct morphology with

minimal stress to the cells and is probably the only method to derive new lines when there are only a few pluripotent cells and the colony needs to be dissected to avoid differentiation. However, the operator should have appropriate experience because it is also possible to select for aneuploid cells that have a growth advantage as they are quicker to form good-sized and "good-looking" colonies. On the other hand, a skilled operator may be able to rescue an aneuploid culture by carefully picking colonies of the correct morphology [69]. Of note, extra care must be taken to avoid damaging cells when using mechanical dispersion on hESC grown in a feeder-free system as these colonies are more fragile than those grown in a feeder-based system. Mechanical passaging is very labor-intensive, and for large-scale culture, as required for manufacturing of differentiated cells for therapeutic use, enzymatic dissociation of colonies into single cells and small clumps is much more efficient. Collagenase, accutase, trypsin, TrypLE (Invitrogen), and EDTA are commonly used for enzymatic dissociation of hESC, but all of them can contribute to clonal aneuploidy at later passages, as single cells and small cell clumps (potentially containing aneuploid cells) are distributed throughout the cell population. The tendency of hESC to undergo clonal aneuploidy reinforces the importance of routine karyotyping while subculturing hESC. G-banding with examination of a minimum of 20 cells or fluorescence in situ hybridization with probes to chromosomes 12 and 17 of a minimum of 200 cells are appropriate karyotyping methods as the gain of an extra copy of chromosomes 12 and 17 commonly gives aneuploid ESCs a growth advantage [70–72].

Nuances of human embryonic stem cell culture

Almost every culture of hESC will contain some differentiating cells, and if the colonies are allowed to overgrow, become multilayered, and/or touch each other, they will begin to show massive spontaneous differentiation within hours. Fig. 23.1 shows the morphology of hESC grown on feeder cells and feeder-free on Matrigel in undifferentiated and slightly differentiated states. Once the cells begin to differentiate, it is very difficult to isolate self-renewing cells. Hand-picking may be used in an attempt to isolate nondifferentiated colonies yet even normal looking colonies may harbor cells committed to differentiation; they may have already lost Oct-4 expression and may even express early differentiation markers like nestin (Fig. 23.3).

Other nuances of hESC culture come to light with experience, and it has been observed many times that different culture conditions may predispose hESC to one or another differentiation route or make a routine differentiation method more difficult to perform. For example, embryoid body (EB) formation is a common first step in many differentiation protocols, and it involves plating a suspension of freshly harvested hESC into plates with low adherence properties where they can aggregate into clumps and differentiate into cells of all three germ layers. EB formation occurs quite readily when hESCs have been growing on feeder layers: multitudes of large, round EBs form in suspension with little cell death. Yet, when hESCs have been cultured in a feeder-free system; EB formation is problematic, the EBs that form are much smaller and sparser, with a lot of cell death observed in the culture. In addition, our experience with multiple hESC lines has shown that some slow growing lines are difficult to maintain due to spontaneous differentiation yet they often show very good yields of derivatives when specifically directed to differentiate. On the other hand, other hESC lines are fast-growing and easy to maintain but do not differentiate as robustly. Given these types of peculiarities, differentiation protocols should be tested and modified when different ESC culture conditions or different hESC lines are used.

Directed differentiation

While spontaneous differentiation of hESC in an adherent state or via EB formation may produce certain types of derivatives (e.g., cardiomyocytes, adipocytes, RPE, and neurons), in many circumstances, a higher yield or purity is desirable. Various bioactive factors and/or selective growth conditions can be employed to direct hESC differentiation toward a particular lineage and/or enrich for a desired cell type. While theoretically, hESC can give rise to all cell types of a human body, in reality, some derivatives are easier to produce in culture than other cell types and some have a higher therapeutic potential than others. As we discuss later, several derivatives have already entered human clinical trials. Initial results from such studies have shown that PSC derivatives from both hESC and iPSC are safe and well tolerated, yet it remains to be determined which therapies may show significant efficacy in clinical trials and be allowed to proceed to commercialization.

The majority of hESC-based clinical trials to date have focused on the use of RPE for the treatment of AMD. The eye has many advantages for testing a first-in-man cellular therapy, including an immunoprivileged status, its small, locally contained structure, ability to noninvasively image the transplantation site, and the easy functional readout of visual acuity [73]. RPE provides "life support" to the photoreceptor cells by supplying nutrients, and among other things, removing shed photoreceptor fragments by phagocytosis, and its malfunction

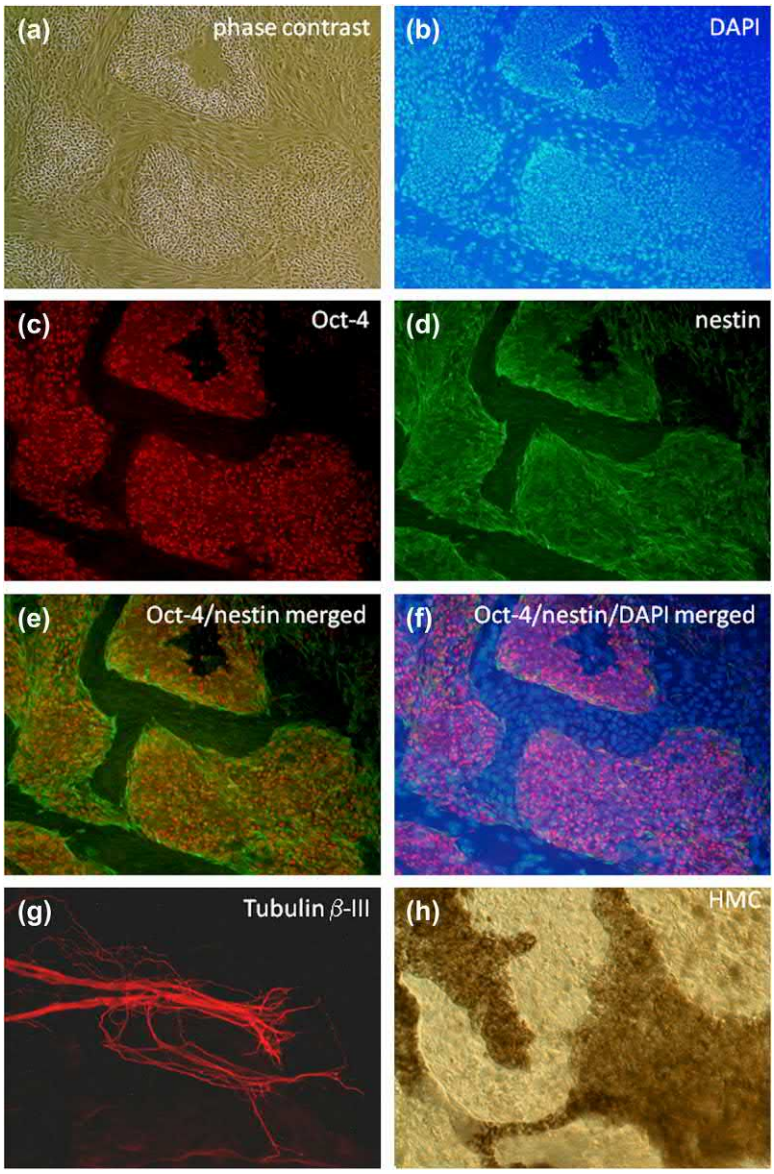

FIGURE 23.3 Spontaneous differentiation of hESC. (A–F)—nestin-expressing cells are seen within Oct-4-positive colonies. (A)—Phase contrast, (B)—DAPI costaining, (C)—Oct-4, (D)—nestin, (E)—Oct-4/nestin merged, (F)—Oct-4/nestin/DAPI merged, (G)—tubulin β-III staining showing a differentiated neuron, (H)—a cluster of RPE. Magnification: (A—F) ×100, (G, H) ×200. *hESC*, Human embryonic stem cell; *RPE*, retinal pigment epithelium. *Images of Oct-4/nestin are courtesy of Jennifer Shepard.*

leads to photoreceptor degeneration and eventually blindness. RPE has several unique features that allow this cell type to be an attractive hESC derivative for regenerative medicine purposes:

1. It is easy to detect in culture and isolate with a high degree of purity. Differentiated RPE cells appear as pigmented clusters visible by the naked eye among spontaneously differentiating hESC after several weeks. Their morphology is very similar to that of cultured human RPE from in vivo sources—polygonal cells with different degrees of pigmentation. When RPE originating from hESC was first discovered in long-term differentiating cultures of hESC, it was due to its unique morphology. Clusters of pigmented epithelial cells were observed and mechanically isolated, expanded, and then confirmed to be of this cell type by the molecular marker analysis [45,74]. This "mechanistic" approach is still being successfully used for isolation of RPE from differentiating cultures of hESC containing multiple layers of multiple cell types, RPE being a minor fraction among other derivatives. When such differentiated cultures are dissociated, it is possible to establish pure cultures of hESC-RPE using cell morphology as the only guidance throughout the mechanical hand-picking of pigmented clusters because, although these cells are mature and differentiated, they are nevertheless fully capable of multiple divisions. One six-well plate of

differentiating hESC can provide enough RPE at passage 2 to treat 10−20 patients (including many more millions of cells for quality testing).

2. The fully differentiated cells retain their proliferative potential for several passages, allowing one to scale up production without any loss of functional properties. This is owed to a distinctive feature of RPE called transdifferentiation. During this process, cells of a differentiated phenotype are capable of partial dedifferentiation and redifferentiation for multiple passages. Under proliferation-promoting conditions, mature RPE cells lose their pigmented epithelial morphology and turn into neural progenitor-like cells (as if going back to their neuroectoderm developmental roots). This is accompanied by the downregulation of mature RPE markers such as bestrophin, CRALBP, RPE65; loss of cell polarity and cuboidal morphology; and upregulation of neural and retinal progenitor markers—for instance, nestin, Pax6, Sox2, and tubulin beta III. When the cells reach confluence and their proliferation slows down, they redifferentiate into pigmented epithelial polarized cuboidal cells and upregulate RPE markers, and if passed again, the cycle would continue for several passages before they senesce and stop dividing or redifferentiating [45,74]. The ability of these fully differentiated cells to proliferate for several passages while maintaining their phenotype and capabilities after they redifferentiate each time is critical for the large-scale production, which is needed for both animal safety studies and human clinical trials.
3. hESC-RPE cells can be frozen and thawed without any loss of functionality and can be maintained in culture in a fashion that ensures optimal conditions for posttransplantation survival and functional engraftment. Thus once mature RPE cells are isolated and plated at passage 0, it becomes possible to monitor and control the degree of their differentiation at each passage. Cells with optimal pigmentation and at the optimal differentiation stage can be identified with differentiation markers and produced for the final harvest. Our studies showed that there was a correlation between the adhesion/survival of RPE in vitro and the degree of maturity/melanin content [48] as well as between their effectiveness in animal models and their degree of pigmentation [45].

The first patient to be given hESC-RPE was in 2011 with a cellular suspension of RPE differentiated from the aforementioned single blastomere−derived, MA09 hESC line. In the United States alone an additional 18 AMD/Stargardt's patients were treated with MA09-RPE, and results from Phase 1/2 trials that have been reported out to 22 months showed the therapy is well tolerated with no evidence of hyperproliferation, tumorigenicity, or rejection—even after transient immunosuppression was discontinued [47]. Additional studies with MA09-RPE [50] and RPE derived from other hESC lines have provided additional evidence supporting the safety and tolerability of cellular suspensions of hESC-RPE [75]. Cellular sheets of hESC/iPSC-derived RPE, either by themselves or on two different synthetic scaffolds, have also been transplanted into AMD patients without any signs of adverse effects [76,77].

In addition to RPE, another PSC-derived ocular cell type, iPSC-corneal epithelium, has recently entered clinical stage testing for corneal disease and injury [78]. Preclinical evidence in a rabbit model of corneal epithelium deficiency shows the transplanted cells restored barrier function. Their generation involves differentiation of a self-formed ectodermal autonomous multizone structure, followed by isolation and expansion of corneal epithelium-like stem/progenitor cells [79]. The group plans to transplant them as a thin sheet into human patients as an alternative to keratoplasty, or the grafting of donor-derived cornea tissue, which is often in short supply. Although iPSCs are being used as a starting material in this study, hESCs could be used in a similar manner.

Ectoderm is often considered to be a default differentiation pathway for hESCs, therefore ectoderm-derived retinal and neuronal cell types have been popular choices for clinical development. As discussed later, several nonretinal, hESC/iPSC-based neuronal cell therapies have begun clinical trials. In fact, the very first hESC-based clinical trial in humans involved testing of oligodendrocyte precursors to treat spinal cord injury. Geron initiated a trial in 2010 for their hESC-oligodendrocyte precursor cell therapy and had treated five patients before funding issues caused them to abandon the trial [80]. Three years later, Asterias Biotherapeutics acquired the asset, rebranded it, and reinitiated Ph1/2a trials. A recent press release from Asterias described safety findings from the trial, which tracked a total of 30 patients for at least 12 months [81]. They reported no adverse effects due to the treatment, and patients either experienced no further decline in motor function or an improvement in motor function recovery following administration of the cells.

Four more PSC-derived neuronal cell types have also reached clinical trials. One of these is another neuronal precursor cell (NPC) type, differentiated from iPSCs, to treat spinal cord injury which has recently received approval to begin clinical testing in Japan [82]. These iPSC-NPC showed therapeutic benefit in a nonhuman primate model of cervical contusion/compression spinal cord injury; they spared motor axons and promoted axonal regrowth, enhanced angiogenesis, prevented demyelination, and improved motor function in behavioral readouts

compared to vehicle controls [83]. A second, neuronal cell type, hESC-derived astrocytes has also been approved to begin clinical trials in Israel in patients with amyotrophic lateral sclerosis (ALS) (NCT03482050). To generate this cellular therapy, hESCs were first differentiated in suspension cultures form to glial-restricted progenitors. Addition of epidermal growth factor (EGF) promoted the formation of neurospheres, followed by adherence and passaging on laminin in the presence of bFGF and EGF to give rise to cryopreservable astrocyte progenitor cells (APCs). These APCs could be further differentiated into committed astrocytes, termed hES-AS, through subsequent 7−28 days of culture without bFGF/EGF. These hES-AS improved survival, slowed disease onset, and improved motor activity in the hSODG93A rat ALS model [84]. In the third recently approved clinical application of neuronal lineage cells, researchers will be transplanting iPSC-derived DA progenitors into patients with PD [85]. In preclinical testing, these cells provided functional improvement in an MPTP-induced nonhuman primate model of PD [86]. In a related study in macaque monkeys, it was found that major histocompatibility complex (MHC) matching of allogeneic DA progenitors (derived from macaque iPSCs) suppressed the recruitment of microglia and other lymphocytes to the graft and thus increased the survival of the cells versus unmatched controls [87]. Such observations suggest that even in an immunoprotected environment such as the CNS, the use of immunocompatible cells may help improve the survival of the graft. The fourth recently approved neuronal lineage trial also involves the use of neural precursor cells, derived from hESC to treat PD (NCT03119636). As mentioned previously, this trial utilizes a parthenogenetic hESC line as the starting material to generate midbrain DA neurons [32]. It will be interesting to follow the clinical testing progress of these various neuronal lineage hESC/iPSC-based therapies to determine which one(s) are most efficacious.

Mesenchymal stromal cells (MSCs), derived from hESCs/iPSCs are a mesoderm-lineage cell type being pursued in clinical trials. MSCs are plastic-adherent, fibroblast-like cells that can be isolated from various tissues including bone, fat, and umbilical cord or derived through directed differentiation of hESC and iPSCs. MSCs secrete a panopoly of growth factors and cytokines, which may impart therapeutic immunomodulatory, antiinflammatory, and trophic support to cells within the body. Several hundred clinical trials are currently underway with adult-derived MSC for treating cardiovascular disease, autoimmune disorders, neurologic disorders, bone and cartilage injuries, asthma, diabetic wounds, and many others [88]. An emerging theory is that younger tissue sources such as cord blood, Wharton's jelly, and hESC may be a better source material for MSC than adult tissues as their youthful nature facilitates greater in vitro expansion and preservation of therapeutic function [49,89,90]. Various preclinical studies have shown MSCs derived from hESCs and/or iPSCs have therapeutic properties in models of, for example, lupus, uveitis, multiple sclerosis, Crohn's disease, hindlimb ischemia, and graft versus host disease [49,91−96]. Three clinical trials are currently testing hESC/iPSC-derived MSCs. This includes Cynata's iPSC-MSCs for the treatment of graft versus host disease (NCT02923375). The company has reported their iPSC-MSCs demonstrated safety and tolerability in a Phase 1 study of 15 treated patients and planning for Phase 2 studies is underway [97]. Two additional studies in China are also underway, where hESC-MSCs are being tested in patients with meniscus injuries (NCT03839238) or primary ovarian insufficiency (NCT03877471). Another mesoderm-lineage PSC derivative that has recently been cleared for clinical trials is Fate Therapeutics' iPSC-derived Natural Killer cell therapy to treat solid tumors (NCT3841110). In November 2018 Fate announced their investigational new drug (IND) application had been approved by FDA [98], and the Phase 1 trial is currently enrolling patients. This represents the first hematopoietic lineage cell type derived from PSCs to reach clinical stage testing. It will be interesting to see which other blood cell type(s) may be next.

Type 1 diabetes and heart disease are two major disease areas for which hESC/iPSC-derived therapies are also being explored. For diabetes the company Viacyte is in Phase 1/2 trials to test their combination product, which consists of glucose-responsive, insulin-producing, hESC-derived pancreatic endoderm cells encapsulated in a delivery device. They reported the first version of their combination product, termed VC-01, was safe and well tolerated out to 2 years, yet the cells did not survive that long in all patients [99]. A newer version of the delivery device has been developed in an attempt to improve long-term viability of the encapsulated cells. This new device, in combination with pancreatic endoderm cells, is termed VC-02, and currently being tested in Phase 1/2 clinical trials (NCT03163511). Viacyte has also entered a collaborative agreement with Crispr Therapeutics to develop a gene-edited version of their allogeneic cell-based therapy in order to bypass the need to encapsulate their cells yet avoid immune rejection [100]. Other groups are also developing PSC-derived pancreatic progenitors or even mature pancreatic beta cells, yet these therapies are still in preclinical development [101−104]. Ischemic heart disease is the other major disease area/large market where hESC/iPSC-based regenerative medicine therapies have reached clinical stage testing. In 2013 Philippe Menasche's group received approval to begin testing his CD15 + Isl-1 + hESC-derived cardiovascular progenitors in a Phase 1 trial (NCT02057900). Six patients were given

a fibrin patch containing 5−10 million progenitors, with epicardial delivery of the patch occurring during coronary bypass procedure. Patients were followed for up to 18 months with no evidence of treatment related adverse events, although one patient died from unrelated comorbidities shortly after the procedure and another patient died of heart failure 22 months posttreatment [105]. Nonetheless, the demonstrated safety and tolerability of this first-in-man study has prompted efforts to test efficacy in Phase 2 studies. A group based in China has also recently launched a clinical study to determine the safety, feasibility, and efficacy of iPSC-derived cardomyocytes (NCT3763136). Several other groups are at the preclinical stage of development for hESC/iPSC cardiac lineage cells to treat heart disease. Recent reports have focused on refining methods for generating more mature cardiac cells from hESCs/iPSCs [106] or trying to understand the basis for and ways to limit arrhythmias associated with grafting of hESC-derived cardiomyocytes in nonhuman primates [107].

Safety concerns

Safety is the number one requirement for the clinical use of cellular products. For hESC derivatives, this means that they have to be nontumorigenic. They must be free of pluripotent cells, which can form teratomas in vivo, and they must show no adverse effects in animal models. They should also be able to survive long enough to be effective after transplantation but not proliferate uncontrollably, although some proliferation followed by a steady quiescent state would be permissible and in certain cases even desirable. This may not be attainable for every derivative of hESC because for clinical use, large numbers of cells need to be generated in culture first. This means that cells of a given type should be able to proliferate in culture but nevertheless maintain a differentiated phenotype after transplantation. This requirement would only allow one to use either fully differentiated cells, which are still capable of multiple population doublings (like RPE), or progenitor/stem cells, which are already committed to a known lineage and do not "misbehave" upon transplantation.

All cell products for clinical use need to be manufactured under good manufacturing practices, pass sterility and mycoplasma tests, and be free of known animal and human pathogens. The presence of animal products during derivation and maintenance of hESC and their progeny could be unavoidable in many cases, but even coculture with live animal cells is not forbidden by FDA regulations. The requirements vary for different countries though. The use of animal cells or products adds more stringency testing of the source cells and their differentiated products. Extensive testing for animal viruses typical for the species whose products were used during manufacturing is required. Special guidelines exist for a xeno product—human cells that have been cocultured with live animal cells—while cells manufactured in the absence of live animal cells and only exposed to animal products are not considered a xeno product. All bovine sera need to come from spongiform-free sources, typically from the United States, New Zealand, and Australia, and gamma-irradiation adds another level of viral clearance.

As previously mentioned, known spontaneous aneuploidy of hESC in culture can occur quite readily and this presents an additional hurdle during manufacturing. Cells need to be frequently monitored for chromosomal aberrations. In addition, any and all hESC derivatives need to undergo extensive testing in animal models to exclude any probability of tumor formation or other serious adverse effects. Such studies should be performed in immune compromised or immune suppressed animals to allow long-term survival of the donor cells. Ideally, the cells should be present in the host for long term, in some cases—throughout the lifetime of the animal; if they proliferate soon after transplantation, they should later become quiescent and stay negative for proliferation antigens, such as Ki67. They should maintain a normal phenotype, which could be assessed by double staining animal tissues with antihuman antigen (nuclei, mitochondria) and antibodies to specific cell markers, and cause no adverse effects including neoplasia. The cells should be able to perform their function in order to justify their transplantation in people. For example, Advanced Cell Technology's MA09hESC-derived RPE met all these safety requirements in long-term studies (9 months) in mouse and rat animal models [45] the cells integrated into the host's own RPE layer, provided photoreceptor support in the models of retinal degeneration (RCS rat) and Stargardt's disease (Elov4 mouse), showed no proliferation beyond the initial 3 months, had normal RPE morphology (cuboidal pigmented polygonal cells), and showed the late RPE marker bestrophin organized in a basolateral, and typical for RPE, in vivo fashion.

Low-level genetic abnormalities or the presence of residual PSCs is a common risk for all PSC-based therapies. To safeguard against the formation of ectopic tissue or tumor formation, hESC/iPSC lines may be engineered with an inducible suicide gene to facilitate elimination of their derivatives after transplantation, if needed. Genetic engineering may be used to provide greater control over the fate of these cells upon transplantation into humans. For example, engineering PSCs with herpes simplex virus type 1-thymidine kinase can induce cytotoxicity of cells exposure to ganciclovir. This may be done by inserting the transgene into a safe harbor locus such as AAVS1 [108] or by tethering its expression to a gene required for cell division, as recently reported [109]. Inducible capsase 9 is another suicide gene that may be used to control

persistence of transplanted cells [110]. In addition to safeguarding PSC derivatives with suicide genes, the issue of potential immunogenicity is a shared risk for application of hESC or iPSCs that are to be used in an allogeneic, off-the-shelf manner. Encapsulation technology, with a variety of different biocompatible materials, has been around for several decades, yet improvements in the technology have garnered renewed hope for its use in protecting transplanted cells against immune responses [111]. As mentioned earlier, Viacyte has been optimizing a semipermeable, biocompatible encapsulation device for their hESC-pancreatic endoderm cells in recent clinical trials. Other recent preclinical studies have focused on the use of alginate derivatives to encapsulate hESC-derived beta cells [104]. An alternative approach that is gaining traction is to genetically engineer cells to make them more immunocompatible. Gene editing technologies such as AAV and Crispr-cas9 are being used to knockout expression of HLA class I and/or class II molecules and overexpress immunosuppressive molecules such as HLA E and CD47 [112–114]. Continued development of such technologies may one day allow hESC/iPSC-based therapies to be used in any organ system with increased safety and reduced risk of immune rejection.

Conclusion

hESCs offer the unique possibility of generating a wide variety of differentiated cells for tissue engineering "from scratch." Their immortal nature allows the production of hESC in virtually unlimited numbers, enabling the creation of a master cell bank and, together with the ability to produce differentiated derivatives, makes it possible to generate the desired cells on a scale large enough for both treating patients and performing all in vitro and in vivo tests necessary to ensure the safety and functionality of these cells. Such derivatives may include progenitor/tissue-specific stem cells capable of differentiating further in vivo (which may offer advantages in tissue repair) and also mature differentiated cells which are still capable of proliferation. The derivatives, when cultured in vitro from an hESC origin, should be maintained in a manner that ensures their optimal in vivo performance. In the coming years, we will hopefully see more hESC-based therapies move from bench to bedside, not only for a range of diseases caused by tissue loss or dysfunction, but as an unlimited source of cells to replace more complex tissues such as skin, bone, and blood vessels, and eventually whole organs such as kidneys and hearts.

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Chapter 24

Induced pluripotent stem cell technology: venturing into the second decade

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Disease modeling

A critical prerequisite for developing therapeutic strategies is to understand the pathological mechanisms of relevant diseases. Animal models have been instrumental in understanding human diseases in an in vivo setting. However, species differences have hindered the full representation of human disease phenotypes in animals. Therefore there is a need for improved models that could better recapitulate the pathophysiology of patients to complement studies in animal models. Primary patient-derived cells are surrogate models that are patient relevant, but primary cells from patients, especially cells from organs such as brain and heart, are not easily accessible. Moreover, mature primary cells such as neurons are not easily expandable. Human-induced pluripotent stem cells (iPSCs) provide an excellent model system for human diseases because of their patient origin, easy accessibility and expandability, and their ability to give rise to nearly any cell types. Moreover, because iPSCs can be derived from individual patients, they can be used to identify patient-specific therapeutic targets for developing personalized medicine [1−4].

For disease modeling using patient iPSC−derived cells, iPSCs derived from healthy individuals were commonly used as a control for patient iPSCs. In addition, the genome-editing technologies have allowed us to generate isogenic iPSC lines that are genetically matched to patient iPSCs but have the disease-causing gene mutations corrected in patient iPSCs, or introduced into control iPSCs. Thus the isogenic control iPSCs will have the introduced mutation as the only variable, allowing the identification of true pathology reliably without the confusion with phenotypes resulted from possible individual-to-individual or line-to-line variations. It is essential to include isogenic iPSC controls when modeling sporadic or polygenic diseases, because phenotypic differences in these situations are expected to be small [5], which can be masked by individual-to-individual or line-to-line variations.

Among the genome-editing technologies, including editing using the zinc-finger nuclease [6,7], the transcription activator−like effector nucleases [8−10], and the CRISPR/Cas9 system [11−14], the CRISPR/Cas9-based editing has been widely used because of its simple design and ease of use. The unmodified Cas9 has been used to achieve gene knock in and knockout [15,16]. The catalytically dead Cas9 (dCas9) fused with a cytidine deaminase enzyme can directly convert cytidine to uridine without double-strand DNA break [17]. dCas9 fused with transcriptional activators or suppressors can activate or suppress gene transcription to induce CRISPR activation or CRISPR interference [18]. Modifications of the CRISPR/Cas9 system also enable mono-allelic or biallelic editing with high precision [19]. The rapidly developing field in CRISPR/Cas9 editing will further facilitate gene editing in human iPSCs and enhance iPSC applications.

iPSC-based modeling is ideal for studying early-onset diseases [20,21], because iPSCs are considered phenotypically young [22]. The relative immaturity of iPSCs and their derivatives may allow them to better recapitulate phenotypes in diseases with an early onset [22]. Modeling late-onset diseases using iPSC-derived cells is more challenging, because aging is an important player in the pathogenesis of late-onset diseases. Different ways are

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exploited to induce cellular aging in order to better model late-onset diseases using iPSC-derived cells. One way is to treat iPSC-derived cells with cellular stressors, including small molecules that affect mitochondrial function, such as pyraclostrobin, or compounds that modulate protein degradation pathways, such as MG-132 [23–26]. Another way is transgenic expression of factors that trigger premature aging, such as progerin [27]. Moreover, direct reprogramming one type of somatic cells to another does not go through the iPSC stage, therefore, could maintain cellular aging markers [28]. Indeed, neurons directly reprogrammed from fibroblasts of aged individuals maintained cellular age [29]. Therefore directly reprogrammed cells could provide a complementary platform to model late-onset diseases.

iPSCs are ideally suited for studying genetic diseases because the disease-causing mutations can be genetically corrected by gene editing to generate isogenic iPSC lines for direct comparison with patient iPSC lines. However, majority of the patients have sporadic diseases, for which the genetic causes have not been identified. For example, about 95% of Alzheimer's disease (AD) patients have sporadic AD. iPSCs can also be used to study sporadic diseases, but it is more challenging, because the phenotypes in such diseases are speculated to be caused by multiple genetic risk variants together with environmental factors. The effect of each variant can be small. The phenotypes in iPSC-derived cells from patients with sporadic diseases are expected to be milder than those from patients with monogenic diseases. Despite the envisioned difficulties in modeling sporadic diseases using the iPSC platform, the development of genetically matched isogenic iPSC lines in combination with allele-specific assays has allowed dissection of a genetic risk variant for sporadic Parkinson's disease [30]. Human iPSC–derived cells have also been used to model sporadic AD to better understand the pathogenesis of this disease [31–48]. Such an experimental paradigm is applicable to model other sporadic diseases.

In the past, iPSC-based disease modeling has taken advantage of a single cell type that is disease relevant. For example, iPSC-derived neural progenitor cells or neurons have been used to model schizophrenia [49–56]. Nowadays, more and more studies have included more than one cell types in iPSC-based disease modeling, including cocultures and 3D organoids. For example, cocultures of astrocytes and neurons have been used to study astrocyte functions [57] and to model the pathology of neurological diseases, such as amyotrophic lateral sclerosis [58–61] and AD [43], while cocultures of astrocytes and oligodendrocytes have been used to model leukodystrophy in Alexander's disease [62].

In addition, the network between different cell types can be modeled using 3D organoids. Organoids generated from human iPSCs have been used to mimic various organs for disease modeling [63–79]. More than one cell types can be generated in an organoid in a spatial–temporal order that follows the developmental program in human. The organoid models are powerful in that they can offer the opportunity to study cell–cell interactions in a cellular context that resembles endogenous organs in cellular organization and structure. The 3D organoids allow disease modeling in a physiologically relevant cellular context.

While organoids have been increasingly used for iPSC-based disease modeling, the technology has its own limitations. One hurdle is to create an organoid with high reproducibility [80]. Using miniaturized spinning bioreactors with 3D design has improved reproducibility in generation of forebrain organoids [75]. A recent study showed that prepatterning, especially dorsal patterning, enhances consistency in both shape and cellular composition of brain organoids [81]. While self-patterned whole-brain organoids exhibited big variations, the dorsally patterned brain organoids displayed much improved reproducibility in organoid shape and cell type collection [81]. The development of more standardized culture conditions, including culture medium, extracellular matrix, and patterning condition, may facilitate the generation of organoids with increased consistency for more precise disease modeling [82]. Another challenge is the lack of vascularization in the current organoid system [83]. Accordingly, organoids exhibit limited growth and maturation due to the lack of continuous nutrient supply. Spinning bioreactors and shaking culture platforms have been shown to provide better nutrient supply and improve the growth of organoids [75,84]. Coculture with endothelial cells has allowed the generation of vascular-like network in organoids [64]. Recently, blood vessel organoids were generated from human iPSCs and embryonic stem cells (ESCs). These organoids contain endothelial cells and pericytes and are able to recapitulate the structure and function of human blood vessels [85]. In addition to optimizing the protocol for organoid development in order to generate blood vessels from cells within the organoids, transplanting human organoids generated in vitro into animals could facilitate vascularization of organoids [86]. The in vivo environment could also help organoid maturation [86].

Drug discovery

Screening for efficacy. Targets believed to be related to disease mechanisms form the basis of classical drug screens. However, as compounds resulting from target-based screening have been unsuccessful frequently, phenotypic drug screening is attracting increasingly more interest [87]. The discovery of iPSCs is strongly aligned with this new interest for a number of reasons, which include (1) drug responsiveness in patient iPSC-based

models that are not experienced using cancer cell line models; (2) the fact that iPSC production is scalable and thereby supports assay development; and (3) the pluripotency of iPSCs, by which difficult-to-assess, disease-related cell types can be differentiated [88]. If the gene responsible for the phenotype(s) is known, gene-editing methods can confirm whether the cellular or molecular phenotype chosen as the readout for drug screening from iPSC-derived cells is actually related to the disease. It is worth noting that phenotypic drug screening does not rule out target-based screening, and that iPSCs are also applicable to target-based screens. In addition, patient-derived iPSC models with either screening type may enable the prediction of responder patient groups according to drug response in a culture dish, which is the so-called in vitro trial [89]. Such in vitro trials can be interpreted as clinical trials in a dish, and they might in fact represent a gateway toward the success of future clinical trials. In this regard, in vitro trials are of major importance, as they can be applied to drug responsiveness testing in a wide-range patient population.

Specific cell surface markers [90,91], cell-specific promoters [92], and microRNAs [93] were established by purification and enrichment technologies in order to obtain a large number of high-purity target cells. For example, iPSC-derived cells have been used to screen drugs for familial dysautonomia, a monogenic early-onset disease highlighted by neuron degeneration in the sensory and autonomic nervous systems. iPSCs from patients with familial dysautonomia were differentiated into neural crest precursors for autonomic neurons, which were sorted and purified and used in the first reported study of large-scale drug screening using an iPSC-based disease model [90]. Splicing defect and production of a dysfunctional truncated protein are the results of this disease, which is caused by mutations in the gene coding for the IkB kinase complex-associated protein (*IKBKAP*). Screening was performed with 6912 compounds, and disease-specific aberrant splicing in the iPSC model was observed to have improved with a compound known as SFK-86466. This compound, however, had no effect on nontarget cells, such as iPSCs, fibroblasts, and lymphocytes. It is clear that the use of iPSC-based drug screenings is advantageous for the analysis of cell type−specific pathogenesis.

In order to avoid cell quality variations, a shorter differentiation period is desirable. In this sense, drug screening using cells obtained from direct reprogramming could offer an alternative approach [94,95], because direct reprogramming usually takes shorter time. Target somatic cells (e.g., fibroblasts) are forced to express cell type−specific transcription factors and one somatic cell state is reprogrammed to another one by direct conversion without passing through the iPSC state [28,95]. Myocardial, liver, and neural cells have been reprogrammed from other type of somatic cells, such as fibroblasts, by direct conversion. Authentic human neurons reflecting vital aspects of cellular aging can be generated by direct conversion [29]. However, as the source of cells provided by this approach is not renewable, it may not be practical to use them for large-scale drug screening.

Patient iPSCs might also be differentiated much more rapidly by the forced expression of transcription factors. By screening 1416 compounds using the survival rate of motor neurons derived from these transgenic iPSC as an index, 27 hit compounds were identified. Among these, 14 were found to target molecular pathways related to Src/c-Abl in Amyotrophic Lateral Sclerosis (ALS) motor neurons [96]. In a drug screening for AD a tetracycline-inducible system was used to introduce NGN2 into iPSCs to generate cerebral cortical neurons with an almost 100% purity following 8 days of doxycycline treatment. A compound group consisting of 1258 existing drugs was screened, and a compound with low cytotoxicity that reduces the amount of Aβ42 was selected [89].

In total, more than 1000 compounds for several diseases have been evaluated by iPSC-based drug screening [90,97−99], and a number of clinical candidates have been identified [96,100−105] (Table 24.1).

Screening for toxicity. New drug development comes at an enormous monetary cost, particularly in relation to failures, including unanticipated side effects, and especially in late-stage clinical trials [106,107]. Among the side effects, cardiac and liver toxicities are of major concern. As a result, methods that could effectively predict the likelihood of candidate drugs that could cause such serious side effects have received much attention.

Among cardiac toxicities, lethal arrhythmias with QT prolongation account for 21% of the total cardiac toxicities [108]. QT prolongation is an unfavorable effect related to human ether-a-go-go-related gene (hERG) channels, and hERG assay is mainly used for cardiac safety testing because blockage of hERG current is considered to be associated with a deadly ventricular arrhythmia termed torsades de pointes (or TdP). Studies have shown that 40%−60% of drugs that inhibit the hERG channel current do not precipitate QT prolongation [109,110], and false positive results from the hERG assay have been a major barrier to the development of promising drugs. As a preclinical approach, the use of in vitro human ion channel assays, human-based in silico reconstructions, and human stem cell−derived cardiomyocytes have been proposed for the detection of drug-induced electrophysiological cardiotoxicity [111]. It has also recently been demonstrated that multielectrode array assays using human iPSC−derived cardiomyocytes might provide a dependable, cost-effective substitute for preclinical in vitro testing [112] that could be applied for appraising proarrhythmic risk [113]. Studies of myocardial toxicity, particularly lethal arrhythmia, are

TABLE 24.1 New drugs in the pipeline from induced pluripotent stem cell screens.

Candidate drug	Target disease	Mechanism	Formulation	ClinicalTrials.gov identifier
BIIB092	PSP	Blocking or elimination of N-terminal tau	Antibody	NCT03068468
Retigabine	ALS	Activation of voltage-gated potassium channels	Small compound	NCT02450552
RG7800	SMA	SMN2 production	Small compound	NCT02240355
Rapamycin	FOP	mTOR inhibition	Small compound	UMIN000028429
Rapamycin	Pendred syndrome	Stress reduction	Small compound	JMA-IIA00361
Ropinirole	ALS	Neurite protection	Small compound	UMIN000034954
Bosutinib	ALS	Src/c-Abl inhibition	Small compound	UMIN000036295

PSP, Progressive supranuclear palsy; *ALS*, Amyotrophic Lateral Sclerosis; *SMA*, Spinal Muscular Atrophy; *FOP*, Fibrodysplasia Ossificans Progressiva; *mTOR*, mammalian target of rapamycin.

progressing, and the US FDA consortium Comprehensive in vitro Proarrhythmia Assay has been launched to establish a uniform standard for myocardial assays based on iPSCs.

In regard to hepatotoxicity, hepatocyte cell lines or human primary hepatocytes are generally used. However, these models also have limitations, such as cell resources, function deficits from freezing and thawing, and lot-to-lot variations. Human ESC (hESC)/iPSC-derived hepatic cells have recently been generated, and they express functional molecules such as CYP3A4 and uptake of indocyanine green [114], the latter of which responds to known hepatotoxic drugs [115]. Better drug screening may also be obtained from functional 3D liver organ buds [64].

Finally, in terms of the nervous system, currently under development is a platform based on the use of pluripotent stem cells to evaluate detrimental drug effects. Many negative effects related to the nervous system, such as epileptic seizures and psychiatric symptoms, are often encountered in phase 1−2 clinical trials, and their prediction is almost impossible with animal models. For more timely identification of potential neurotoxicity, assays using human iPSC−derived neuron for functional phenotypes can be conducted, similar to TdP for cardiotoxicity.

Stem cell−based therapeutic development

Since the discovery of hESCs in 1998 [116] and the development of human iPSC technology in 2007 [2,3], continuous efforts have been devoted to apply the technologies into cell therapy development. Of the 21 clinical trials conducted currently with hESC- and iPSC-based cellular products, 12 are for ESC- and 2 for iPSC-derived retinal pigment epithelial (RPE) cells to treat macular degeneration (https://clinicaltrials.gov). A list of clinical trials using human iPSC−derived cellular products is summarized in Table 24.2. The first clinical research using human iPSC products was started in 2014. Autologous RPE sheets derived from patient's own iPSCs were transplanted back to the patient [117]. The therapy has led to a favorable outcome, halting macular degeneration without administration of anti-vascular endothelial growth factor (VEGF) drugs. After a temporary hold of the trial, because mutations were found in the second patient's iPSCs [118], another clinical research using allogenic transplantation was started in 2017.

In order to apply human iPSC−derived cellular product into clinical applications more commonly, several hurdles need to be removed [119]. One concern associated with the development of pluripotent stem cell−based cell therapy is the risk of tumorigenicity from these cells [120]. Therefore before clinical applications, iPSC-derived cellular products need to be carefully tested to ensure their identity, purity, and sterility.

It is critical to safeguard the final iPSC-derived product not to contain undifferentiated pluripotent stem cells that have the potential to generate teratomas. Continuous efforts have been invested to develop protocols for differentiating human iPSCs into specific cell types with increased purity. Another solution is to sort the iPSC-derived cells before

TABLE 24.2 Clinical trials using human induced pluripotent stem cell (iPSC)-based cellular products.

Cell type	Cell source	Disease indication	Trial start time	Sponsor	Investigator
iPSC-derived retinal cells	Autologous	Macular degeneration	2014	Riken Institute	Masayo Takahashi
iPSC-derived retinal cells	Allogeneic	Macular degeneration	2017	Riken Institute	Masayo Takahashi
iPSC-derived dopaminergic precursors	Allogeneic	Parkinson's disease	2018	Kyoto University	Jun Takahashi
iPSC-derived mesenchymal stem cells	Allogeneic	GVHD	2018	Cynata Therapeutics	Kilian Kelly
iPSC-derived corneal cells	Allogeneic	Corneal disease	2019	Osaka University	Koji Nishida
iPSC-derived neural stem cells	Allogeneic	Spinal cord injury	Planned for 2019	Keio University	Hideyuki Okano
iPSC-derived cardiomyocyte sheets	Allogeneic	Heart disease	Planned for 2019	Osaka University	Yoshiki Sawa

GVHD, Graft-versus-host disease.

transplantation. Methods have been established to further purify iPSC-derived cells through positive selection for lineage-specific markers and/or negative selection against hESC surface markers, using fluorescence-activated cell sorting or magnetic-activated cell sorting. Serial spiking human iPSCs into iPSC-derived cellular products and then transplanting the spiked products into animal models to test tumorigenicity could also help to predict the risk of tumorigenicity from any residual iPSC population in the final product.

The iPSC-derived cellular product will be generated under Good Manufacturing Practice (GMP) condition and undergo extensive release tests. Cells that meet the specification of all the release tests can be moved forward to clinical trials. After the cellular product is delivered into patients, the patients who received the treatment will be monitored rigorously for the potential of developing any tumors and activation of the immune system [121].

Human iPSCs can be used to generate both allogeneic and autologous cellular products. A major challenge for allogenic transplantation is immune rejection caused by the human leukocyte antigen (HLA) mismatching, which has been coped through immune suppression. From the immunological point of view, autologous transplantation is ideal for cell therapy because they may avoid the high cost and serious side effects associated with lifelong immunosuppression required for allogeneic cell transplantation [122]. Moreover, using a patient's own iPSCs represents the unique advantage of iPSCs over ESCs and constitutes the cornerstone of precision medicine. Accordingly, the first iPSC clinical research used RPEs from autologous iPSCs [117]. The autologous iPSC products could be ideal for the treatment of orphan diseases, for which a master cell bank is not necessary. For more common diseases, autologous iPSC therapy for a large number of patients may not be practical at the present time because of the high cost associated with manufacturing and long time needed for release testing of each cell line. For these reasons a clinical research using allogeneic iPSC-RPE has been started in 2017 in Japan.

To address the concern of potential immune rejection associated with allogeneic transplantation, iPSC stocks derived from HLA homozygous donors are being established for covering most HLA haplotypes in Japan. It is estimated that more than 150,000 donors need to be screened in order to generate 140 HLA-A, HLA-B, and HLA-DR homozygous iPSC lines to cover 90% of the Japanese population [123,124].

Combining genome editing with the iPSC technology to generate universal donor cells creates another avenue to address immune rejection complications associated with allogeneic transplantation [125]. Knockout the β2-microglobulin (B2M) gene eliminates surface expression of class I HLA molecules, thus could inhibit the immune response from cytotoxic T cells. However, these cells can be lysed by natural killer (NK) cells. Knocking in the HLA-E gene at the B2M locus in human pluripotent stem cells led to cells that could evade immune attack by CD8 + T cells and are resistant to NK-mediated lysis [126]. Knocking in the immunosuppressive receptor cytotoxic T-lymphocyte-associated protein 4-immunoglobulin and programmed death ligand 1, both of which inhibit

T cell−mediated immune responses, has also been shown to protect hESC-derived cells from allogenic immune rejection [127]. More recently, depletion of HLA I and II surface expression by knocking out of the B2M gene (encoding a structural component of the major histocompatibility complex (MHC) class I molecules) and CIITA gene (the master regulator of MHC class II molecules), together with overexpression of CD47 (a membrane protein that interacts with cell surface receptors to prevent phagocytosis) in both mouse and human iPSCs, results in hypoimmunogenic cells that can survive in allogeneic recipients without the need of immunosuppression [128]. In a parallel study, allele-specific genome editing of HLA heterozygous donors was used to generate HLA class I pseudo-homozygous iPSCs [129]. Moreover, deletion of both HLA-A and HLA-B but retention of HLA-C led to the generation of HLA-C-retained iPSCs. Cells generated from both approaches could evade the immune response from T cells and NK cells. It was estimated that 12 lines of HLA-C-retained iPSCs, combined with HLA class II knockout, could cover more than 90% of the population worldwide in an immunologically compatible manner, thus greatly facilitating the dissemination of human iPSC−derived cell therapies.

Concluding remarks

The human iPSC technology has provided a powerful tool for us to understand and treat human diseases. The iPSC platform, along with other technologies, such as gene editing, 3D organoids, and direct reprogramming, has allowed us to directly observe and treat patient cells in a dish. Knowledge gained from iPSC-based disease modeling has enhanced our understanding of both normal development and disease pathogenesis, which will help us to design better therapies for patients with relevant diseases. Much progress and excitements await us to witness in the next decade of iPSCs.

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Chapter 25

Applications for stem cells

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Introduction

The capacity for unlimited self-renewal and the potential to differentiate into any cell in the body made embryonic stem cells (ESCs) a valuable research tool. Mouse ESCs, first derived in 1981 [1], have been used for the last 30 years in conjunction with gene targeting technologies to produce genetically modified mice; large-scale federally funded projects, such as the Knockout Mouse Project, have sought to create a comprehensive collection of mice with specific genetic changes [2].

Human ESCs (hESCs), derived in the late 1990s, proved to be a greater challenge; although they were generated from blastocyst embryos using the methods developed to derive mouse ESCs, they proved to be much more difficult to maintain in cell culture, and when the National Institutes of Health began funding hESC research in 2002, one of their first programs was to sponsor seven hESC laboratory courses across the United States. These courses spawned the publication of comprehensive hESC laboratory methods [3] and trained a cohort of scientists, some of whom went on to use the technology in their own laboratories. In 2006 just as hESC methods were becoming standardized and disseminated, there was a sea change that accelerated the nascent field of regenerative medicine: the discovery that adult somatic cells could be reprogrammed into induced pluripotent stem cells (iPSCs). That discovery fundamentally changed the stem cell field, broadly expanding the possible uses of PSCs.

Reprogramming of somatic cells into induced pluripotent stem cells

The production of iPSCs was first reported by Takahashi and Yamanaka using adult mouse fibroblasts in 2006 and was immediately hailed as a ground-breaking discovery [4]. The introduction of four transcription factors, *Oct3/4* (*Pou5f1*), *Sox2*, *Klf4*, and *c-Myc* (*Myc*), also called the "Yamanaka factors," was sufficient to transform adult fibroblasts into PSCs. In 2007 the group repeated the process using human fibroblasts [5]. The process of reprogramming adult cells into cells functionally equivalent to hESCs has provided an unparalleled tool to study human development and generate cells for regenerative medicine applications. Yamanaka's discovery altered the dogma underlying our knowledge of cell biology and created new fields of study. In 2012 Shinya Yamanaka was awarded the Nobel Prize in Physiology or Medicine, along with John Gurdon, for the discovery that mature cells could be reprogrammed into cells capable of developing into all cells of the body. Gurdon is credited as the first to demonstrate that a cell nucleus could be reprogrammed to a pluripotent state through the process of somatic cell nuclear transfer [6,7].

Since the first iPSC study was published, scientists have gained a much better understanding of what was once mostly a black box process, in which the Yamanaka transcription factors were expressed as transgenes in cells and sometime later pluripotent colonies would emerge. Since the advent of iPSC technology the molecular events underlying reprogramming have intrigued researchers. It is important to note that the minutiae of reprogramming events have been almost entirely studied in mouse cells, which can be made from genetically modified mice and are much more easy to manipulate than human cells. There are important embryological and genetic regulatory differences between mouse and human cells, and we caution that the details of molecular events in human cells remain to be understood.

Early work indicated that the reprogramming process in mouse cells is a positive feedback loop by which core

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transcription factors regulate the expression of a network of pluripotency-associated genes [8,9]. Early models postulated that reprogramming is a stochastic process in which most transgenic cells can initiate reprogramming but only a few achieve pluripotency [10,11]. With advancements in single cell gene expression profiling methods, it has become possible to delineate cell types and cell states in order to understand the reprogramming mechanism in molecular detail. Early studies using single-cell approaches at defined timepoints demonstrated that after the initiation of transgene expression, there is a hierarchical phase with *SOX2* as the upstream factor in the gene expression hierarchy, meaning that endogenous *SOX2* expression was required before endogenous expression of other key pluripotency-associated genes [12].

A more recent study examined mouse fibroblast reprogramming by profiling cells at frequent intervals across the first 18 days of reprogramming, using an inducible reprogramming system [13]. Using a mathematical technique called "optimal transport," the researchers were able to temporally couple cells profiled at each timepoint and assign ancestors, descendants, and trajectories. In this study, expression of the Yamanaka factors was withdrawn at 8th day of reprogramming and cell fates immediately bifurcated to two routes: a stromal cell−like state or a mesenchymal−epithelial transition (MET) state. From days 9 to 18, cells that originated from the MET state further branched into three types: trophoblast, neuronal cells, and pluripotent cells. Cells on the trajectory to become iPSCs dropped from ∼40% on day 8.5 to 1% on day 11, suggesting that they go through a tight bottleneck to become iPSCs. Intriguingly, the stromal cells that never became iPSCs could secrete cytokines that promote iPSC formation: for example, GDF9 secreted from the stromal cells appeared to enhance reprogramming efficiency.

Reprogramming itself has been used as a tool to systematically explore the role of various factors in the generation and maintenance of pluripotency. For example, redundancies in the pluripotency network have been identified, such as replacing *SOX2* in the reprogramming process by the closely related *SOX1* and *SOX3* [14]. Kruppel-like factor 2 (*KLF2*) or *KLF5* can be used as substitutes for *KLF4* [15], and MYCL (MYCL proto-oncogene), which has less oncogenic activity, has been used in place of MYC [16]. Multiple research groups have reported that reprogramming is more efficient when the tumor suppressor, p53, is reduced during the reprogramming process (discussed in Ref. [17]).

Epigenetic remodeling

For reprogramming to occur, major epigenetic barriers must be overcome. Epigenetics refers to modifications of the genome that can affect the ability for genes to be expressed. The addition of a methyl (CH_3) group to the five-prime carbon of the cytosine ring is a well-characterized example that typically results in transcriptional inhibition. Throughout the process of differentiation the methylation pattern in the genome changes and stabilizes different cell states, shown most dramatically in vitro by single base-pair DNA methylome sequencing of undifferentiated and differentiated human PSCs [18]. As such, the methylation state of an adult somatic cell must be remodeled during the reprogramming process to unlock access to the pluripotency-associated gene network. Many of these genes are hypermethylated in donor cell types but are hypomethylated in iPSCs [19]. The reprogramming factors do not affect DNA demethylation directly, so modifications of DNA methylation are likely a secondary effect of transcription factor induction. Achieving pluripotency also requires considerable histone remodeling [20].

Some studies have raised concerns that reprogrammed cells retain an "epigenetic memory" of the somatic tissue from which they were originally derived [21,22]. However, studies have indicated that epigenetic differences account for only a small fraction of the variability among iPSCs and ESCs [19,23]. In addition, like the lead character in the movie *Memento* (https://en.wikipedia.org/wiki/Memento_(film)), the epigenetic memories of iPSCs are short-term and easily lost; it has been shown that time in culture reduces the epigenetic differences among iPSCs [22,24]. This suggests that the global epigenetic patterns of iPSCs stabilize over time, although there are enduring hot spots of variation, such as at imprinted regions [19,25]. Detailed analysis of global gene expression and DNA methylation patterns reveals that variability among ESCs and iPSCs occurs largely because of variations among individual cell lines rather than differences among classes of PSCs [19,23,26−28].

Reprogramming techniques

The reprogramming process initially was developed using retroviral transduction. Starting from a candidate pool of 24 transcription factors considered to have important roles in pluripotency, Yamanaka reported that the combination of *Pou5f1*, *Klf4, Sox2*, and *Myc* was sufficient to produce iPSC colonies from adult mouse fibroblasts. Because the first iPSC lines were created with retroviral vectors that integrated within the host genome, there was concern that uncontrolled integration could disrupt tumor suppressor genes or activate oncogenes through the process of insertional mutagenesis. Of additional concern was that one of the Yamanaka factors, Myc, is an oncogene itself and that reactivation of the transgenes could result in tumor formation. Indeed, 20% of the offspring of chimeric mice derived from

retrovirus-reprogrammed iPSCs developed tumors [29]. It has also been reported that human iPSC-derived neural stem cells containing integrated vectors produced tumors in a mouse spinal cord injury model [30].

Because of the potential tumorigenicity of cells reprogrammed with integrating vectors, there is general consensus that the use of reprogramming methods that integrate transcription factors into the host genome is ill-advised if the cells are planned for clinical use. However, tools available to reprogram cells have rapidly evolved, and now a variety of nonintegrating alternatives exist and are widely practiced [31]. Integration-free methods rely on the fact that transcription factors used to reprogram the cells are necessary only during the early stages of reprogramming, after which awakening of the endogenous pluripotent machinery is sufficient to sustain the pluripotent state. The Sendai virus is a commonly used, nonintegrating method of reprogramming (see Fig. 25.1) [32]. This RNA virus does not translocate to the nucleus and is diluted with each cell division. In addition, episomal vectors can be used to deliver the Yamanaka factors; however, it has been demonstrated that episomal vectors can sometimes integrate into the host genome, so iPSC clones must be monitored by genomic analysis [31]. Another popular method for reprogramming is the introduction of synthetic messenger RNAs (mRNAs) [33]. The challenge with mRNA reprogramming is the need to repeat applications [34].

The most widely used somatic cells are skin fibroblasts and blood lymphocytes. Both cell types can be reprogrammed with similar efficiency. The efficiency of the first human reprogramming experiments was less than 1 cell in 100 parental cells, but reprogramming efficiencies of greater than 1% are now common. In general, the methods only need to be efficient enough to produce a few clones of iPSCs; because reprogramming is usually reproducible and much of the effort involved in reprogramming is in confirming pluripotency and characterization of the iPSCs, analysis of more than a small number of clones is not cost-effective.

There are two facts about the production of iPSCs that are initially nonintuitive and therefore important to note. It is important to keep in mind that each iPSC line is derived from a single somatic cell. Both fibroblasts and lymphocytes are mosaic [35], with individual cells differing in genomic sequence, so it is to be expected that multiple iPSC clones from a single individual person would not have identical genome sequence. This may or may not be a problem, depending on whether the genetic variations have developmental or safety consequences.

iPSCs are typically clonally derived, which means that single colonies of reprogrammed cells are picked and subcultured separately from other colonies. Clonal derivation preserves the genetic homogeneity of the iPSC line, a factor that is important in studying genetic disorders, such as trisomy 21, that are variably mosaic, and not present in all of a person's cells. It has been argued that for high-throughput reprogramming, it is necessary to skip the clone-picking step and combine multiple clones [36]. Mixture of clones will lead to fluctuations over time in the subsequent culture population as they are expanded, because some iPSC clones will divide slightly faster or be more resistant to stress than others. Many genetic diseases are also mosaic, so a single blood sample or a culture of fibroblasts will yield both normal and mutant iPSC clones. If clones are combined, they would need to be later subcloned and characterized in order to distinguish the differences.

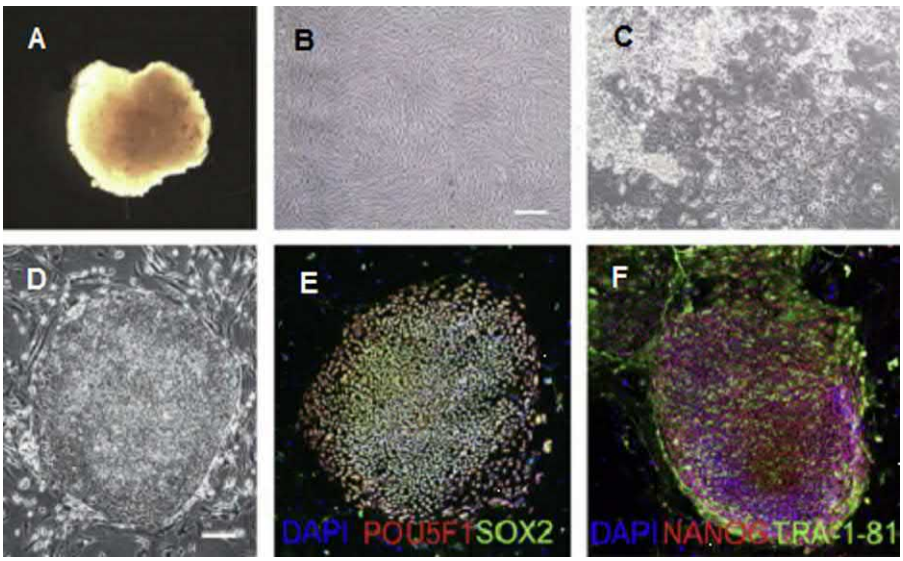

FIGURE 25.1 Reprogramming human dermal fibroblasts using Sendai virus to deliver the four transcription factors. A biopsy of dermal tissue (A) is obtained from the patient. The biopsy is then processed and dermal fibroblasts are isolated and cultured (B). After introduction of the Yamanaka factors, the morphology of the fibroblasts changes (C) as the cells undergo the reprogramming process. Finally, after a period of several weeks, colonies emerge (D) that express pluripotency markers, as shown by immunocytochemistry (E and F). Scale bar 50 μm in (B and C) and 100 μm in (D and F).

Induced transdifferentiation

The same principle of transcription factor–based reprogramming has been applied to direct transformation of one cell type to another. The process of transdifferentiation, also known as lineage reprogramming or direct lineage conversion, can bypass the pluripotent state when converting one cell type to another. Fibroblasts can be transdifferentiated into terminally differentiated cell types, such as neurons [37] or cardiomyocytes [38], without passing through the iPSC stage. Bioinformatic analysis can be used to predict what transcription factors are needed to convert one cell type into another [39], although sometimes the same transcription factor is active in more than one cell type, which leads to cells that are hybrids of two or more cell types [40,41]. Large transcription factor screens have been performed to identify which combination of genes must be induced to be expressed to turn somatic cells into diverse subtypes of neurons [42]. This approach is potentially useful for obtaining mature cell types for which there is currently no robust differentiation scheme, but the cells must still be rigorously characterized. If the products of the transdifferentiation are postmitotic and can be separated from the starting fibroblasts or other cell types, there is less concern about contamination with potentially dangerous proliferating cell types.

There are also drawbacks that should be considered in using direct transdifferentiation of cells such as fibroblasts to mature cell types. Like reprogramming to iPSCs, this process is inefficient, and because mature cell types have a limited capacity to divide (neurons and cardiomyocytes are postmitotic), direct reprogramming requires a large input population and must be repeated each time more cells are required. Transdifferentiated mature populations are not clonally derived, which means that genetic manipulation and characterization of the resulting cell population are difficult or not feasible. These drawbacks may be mitigated by targeting an intermediate progenitor or stem cell that retains a capacity for proliferation but is limited in differentiation capacity; this would allow for purification of the cell type desired and elimination of any abnormally programmed cells [43].

Genomic stability

A study examined the potential for genomic damage during reprogramming using whole-genome sequencing and concluded that the reprogramming process itself is not likely to introduce mutations with adverse side effects [44]. Another exome sequencing analysis of fibroblast and iPSC clones concurred and reported that almost all of the genomic variation in iPSCs originates from the cell population used for reprogramming [45]. However, after reprogramming, populations of iPSCs can drift with time in culture like any other cell type, because genetically aberrant subpopulations can tend to divide more quickly or be more resistant to apoptosis or other forms of cell death. The types of genetic variations observed range from large-scale karyotypic abnormalities to copy number variations and point mutations. Common karyotypic changes in iPSCs and hESCs include trisomies of chromosomes 12, 17, and X, and duplications of subchromosomal regions on these chromosomes [46–48]. In addition, studies of trinucleotide repeat diseases, including Friedreich ataxia and Fragile X syndrome, have reported changes in the repeat length after reprogramming [49,50].

Because iPSCs are self-renewing, they never senesce and will continue to divide as long as they are in culture. Because mutations arise when cells replicate, it is inevitable that genomic abnormalities will accumulate if the iPSCs are cultured for long periods of time. Of particular concern are the appearance of mutations in cancer-related genes such as tumor suppressor *TP53* in hESCs and iPSCs after prolonged time in culture [51,52].

Applications of induced pluripotent stem cells

There has been concern in the field that iPSCs are somehow inferior to hESCs for practical applications. However, a number of reports have demonstrated that hESCs and iPSCs are essentially indistinguishable [19,27,28]. A study on transcriptional and epigenetic comparisons using genetically matched hESC and iPSC lines revealed that hESCs and iPSCs are molecularly and functionally equivalent and cannot be distinguished by their gene expression profiles [53]. This means that all of the applications established for hESCs should be easily transferred to iPSCs (Fig. 25.2).

Disease modeling

Perhaps the most attractive feature of iPSC technology is that it allows scientists to study human diseases using human cells. Much progress in modeling human disorders has been made possible by pioneering work to develop robust protocols to generate specific cell types from human iPSCs, including neurons [54–56] and cardiomyocytes [57]. iPSCs have been used routinely to model genetic diseases, including monogenic disorders and more complex genetic disorders. Animal model systems have been used to study those disorders for decades, but positive results in animals have not always translated to human studies. This should not be surprising because rodents diverged evolutionarily from humans almost 60 million years ago. For example, in the nervous system, understanding the mechanisms underlying neurological

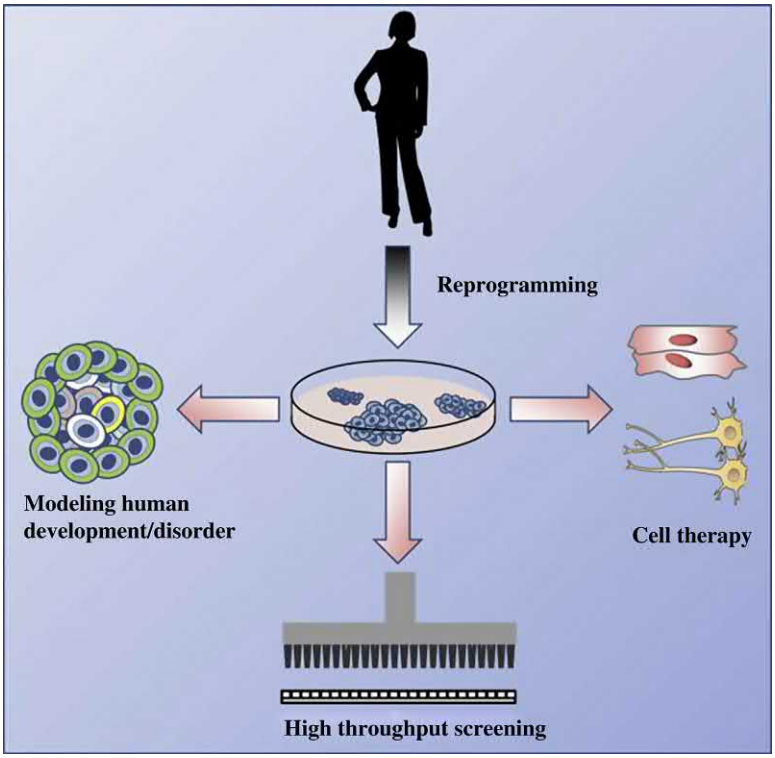

FIGURE 25.2 Applications of iPSCs. Human iPSCs can be used to study and treat human diseases. Aggregates of iPSCs can form organoids that recapitulate aspects of early embryonic development that can be used to model human developmental disorders. Genetically, diverse iPSCs and their derivatives can be used in high-throughput screening to improve the preclinical development of pharmaceuticals. In addition, iPSCs can be used to derive therapeutically relevant cell types for autologous transplantation as a cell therapy to treat human diseases. *iPSCs*, Induced pluripotent stem cells.

disorders and the development of pharmaceutical interventions has lagged far behind disorders affecting other organs. Many highly anticipated drugs, such as those that target Fragile X syndrome [58] and amyotrophic lateral sclerosis (ALS) [59–61], have failed to demonstrate efficacy in human trials after promising preclinical work on animal models. In addition, neurological and psychiatric diseases are complex because they are rarely based on a single gene variant. Notably, many psychiatric diseases arise from mutations in noncoding regions [62,63]. In many cases, these noncoding regions either are not conserved in rodents or they function differently. In contrast, iPSCs derived from patients with genetic disorders contain all of the complex genetic interactions that underlie the disease.

Timothy syndrome (TS) is a neurodevelopmental disorder that has been successfully investigated using patient-derived iPSCs [64]. TS is a rare monogenic form of autism caused by prolonged activation of the L-type $Ca_v1.2$ calcium channel. The prolonged channel activation results from a gain of function mutation leading to dysregulation of $Ca^{2þ}$ signaling in many cell types. One of the striking phenotypes of TS iPSCs is abnormal cortical development; they produce fewer upper layer neurons and a higher proportion of lower layer neurons. Interestingly, elevated $Ca^{2þ}$ signaling leads to the upregulation of tyrosine hydroxylase in cortical neurons. Higher levels of tyrosine hydroxylase, an enzyme that is involved in the biosynthesis of catecholamines, ultimately lead to increased norepinephrine and dopamine production that is associated with aggression in TS patients. Using iPSCs as a model, it was discovered that roscovitine, an L-type channel blocker, can restore normal tyrosine hydroxylase expression and catecholamine production in TS iPSC–derived neurons. Notably, the transcription regulatory element of human tyrosine hydroxylase locus is not conserved in mice, and the catecholamine phenotype is not observed in a transgenic TS mouse model [65]. In this way the TS iPSC model provided preclinical validations for future therapeutic development that would otherwise have been missed using only rodent models. Similarly, studies using iPSCs to model other monogenic forms of autism such as Phelan–McDermid syndrome [66], Rett syndrome [67], and Williams syndrome [68] identified human-specific phenotypes and discovered disease-causing pathways.

iPSCs are also useful for studying diseases for which there is evidence of inheritance but no specific mutations identified. For complex diseases such as idiopathic autism and schizophrenia, most cases lack a clear genetic basis. Deriving iPSCs from the patients preserves their genomes, and when differentiated into the relevant cell types, they can be used to study cellular phenotypes and molecular mechanisms without knowing the genetic cause. However, patients who have complex diseases usually present with a wide range of symptoms. Because

obtaining information about a disease from patient iPSCs requires multiple patients with the same syndrome, careful consideration should be taken in selecting patients and study controls. For example, to study idiopathic autism, a research group selectively focused on patients who presented with the same clinical phenotype: early brain overgrowth. They observed an increased proliferation rate of the disease-associated iPSC-derived neural progenitor cells and determined that it was likely caused by dysregulation of the b-catenin/BRN2 cascade [69]. Selection of appropriate controls is extremely important to minimize nondisease relevant differences; because humans are genetically diverse, variations in individuals' iPSCs could contribute to differences in cellular behaviors that are not necessarily disease relevant. For this reason, iPSCs from unaffected family members are usually used to study idiopathic disease to minimize variability owing to genomic diversity [70].

iPSCs are also valuable for validating human-specific disease-causing variants. Advances in sequencing technology have enabled an increase in genome-wide association studies (GWAS) to identify novel disease-causing candidate mutations. For many complex diseases, candidate mutations are found in noncoding regions and most are not evolutionarily conserved in animal models. iPSCs are an ideal platform for validating such candidate mutations identified through GWAS. An example is a single nucleotide polymorphism risk variant that appeared to contribute to the pathogenesis of Parkinson's disease (PD) [71]. Using genome editing with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), risk-associated alleles were introduced into wild-type iPSCs; by comparing the otherwise identical cell lines, researchers showed that the risk allele altered *cis*-regulation of the alpha-synuclein (SNCA) locus and led to an upregulation of SNCA expression. This variant mimicked a duplication of the coding region for SNCA that was known to be a familial cause of PD [72].

Methods have improved for culturing iPSCs in miniature "organoids" that can resemble the structure of human organs. When iPSCs are aggregated during their differentiation in vitro, they can create their own microenvironments and self-organize into three-dimensional structures. Kidney [73], liver [74], stomach [75], and brain organoids [76] can show remarkable similarities to the corresponding tissues. Researchers have just begun to explore possibilities to model disease using organoids. Multisystem organoids have been developed, including intestinal organoids, with components of the enteric nervous system [77]. Organoids may provide a means to produce more fully mature cells in vitro and may be useful for drug screening.

Challenges and future possibilities in disease modeling

It is difficult to mature cells fully in vitro, so it remains challenging to model late-onset diseases. Efforts have been made to age iPSC-derived cells artificially, such as progerin treatment [78] and telomere shortening [79], but it is not clear whether these treatments are realistic mimics of aging. Direct reprogramming from fibroblasts has been reported to preserve age-related characteristics of the donor [80], but direct reprogramming has shortcomings for disease modeling, as discussed earlier. However, in some cases, aspects of late-onset genetic diseases may be detected in iPSC-derived cells. This allows testing of drugs that can correct the cellular phenotype. In one example, ALS patient–derived motor neurons were found to have an abnormal electrophysiological phenotype, which could be corrected with a US Food and Drug Administration approved anticonvulsant drug, Retigabine [81]. A clinical trial was initiated in 2016 (https://clinicaltrials.gov/ct2/show/NCT02450552) to evaluate the effects of Retigabine on motor neuron activity in people with ALS.

For decades, drug discovery has relied heavily on a reductionist approach in which high-throughput screening is used to identify biologically active molecules based on expression of a recombinant target protein of interest in a single transformed cell line. The transformed lines used in these screening processes tend to be of similar ethnic backgrounds. Decades of experience using this model led to the finding that the genetic background of an individual can determine the success or adverse effects of a particular drug. For this reason, regulatory agencies recommend, including a diverse patient population in later-stage clinical trials. A genetically diverse group of iPSC-derived cells would be instrumental in predicting these types of genetically dependent effects in the preclinical stages, making the drug development process more efficient [82].

Proof-of-concept toxicity studies have been performed in stem cell–derived cardiomyocytes [83], hepatocyte-like cells [84], and neurons [85]. However, to draw associations between genotype and drug response, as well as identify associated biomarkers, large collaborative initiatives are needed to generate cell banks that cover diverse ethnic groups, capturing a range of genotypes [82]. Carefully chosen sets of genetically diverse iPSCs could then be used for preclinical development.

In addition to the ethnicity of the patient, multiple disease mechanisms are likely to contribute to one disease phenotype, and one drug is not appropriate or sufficient for all cases. For example, cystic fibrosis is an autosomal recessive disease that is caused by mutations in a gene called cystic fibrosis transmembrane conductance

regulator (CFTR), which encodes for a chloride channel. Mutations in this channel result in abnormal regulation of salt and water, causing severe respiratory and gastrointestinal symptoms. More than 1900 CFTR mutations have been identified (www.genet.sickkids.on.ca). The type of mutation has a direct effect on the efficacy of commonly used medications. For example, for patients who carry a G551D mutation, CFTR channel activity is reduced. Ivacaftor, an oral agent and channel agonist designed to prolong the time in which the chloride channel is open, was effective in treating symptoms in this subgroup of patients [86]. However, this patient subgroup accounts for only 5% of patients with cystic fibrosis. For most patients a truncation in the protein prohibits proper trafficking to the plasma membrane. In such cases a channel agonist is ineffective. Another therapeutic, lumacaftor, which aids in transporting CFTR to the cell surface, is more appropriate for these patients [87]. Identifying the disease mechanism for each CFTR variant could be enhanced by using patient-specific iPSC modeling.

iPSC model systems have been used to predict the clinical outcome of patients with specific mutations. One study examined a selective sodium channel blocker and its effect on inherited erythromelalgia using patient iPSC−derived sensory neurons and could correlate the in vitro results to patient response and specific mutations [88]. As another example, a retrospective study identified patients who clinically experienced drug-induced QT prolongation upon administration of a nonselective beta-blocker, sotalol. Cardiomyocytes derived from these patients' iPSCs recapitulated the patient response in vitro, demonstrating differential arrhythmias after sotalol treatment [89]. These studies demonstrate the exciting potential for using iPSCs to predict patient outcomes and to choose better from clinically available interventions.

Disease-modifying potential of induced pluripotent stem cells

Cell therapy is a fast-developing field in modern medicine. Traditionally, cell therapy was limited to bone marrow cells and blood stem cells, but with the ability to generate clinically relevant cell types from stem cells, PSC-based cell therapies are being developed. A potential advantage of using iPSC-based therapies is that the cell line can be chosen to be patient-specific or a close immunological match. Autologous transplantation of iPSCs and their derivatives is expected to be tolerated by the immune system, and patients could benefit from avoiding immunosuppressant treatment required for allogeneic transplantation. The creation of banks of iPSCs designed specifically to serve as a close immunological match to a large percentage of the population may also be feasible by deriving cells from individuals homozygous for the major human leukocyte antigen (HLA) markers. Such banks would reduce the time and cost needed to produce cells for therapy. The practicality of these banks depends on the number of cell lines needed to match the population served by the bank. In a country such as Japan, it is estimated that a bank of 50−100 carefully chosen iPSC lines could match 90% of the population [90]. In contrast the genetic diversity of the populations of Europe and the United States would necessitate banks with orders of magnitude more cell lines, which makes the design more challenging and less feasible. The HLA-matched iPSC banks have not yet been tested in human trials, and there is concern that minor antigens could cause rejection even when there is a perfect major HLA match.

The expectation of immune tolerance of autologous iPSC-derived transplants, however, has been brought into question by reports showing that transplantation of mouse iPSCs into syngeneic rodents led to immunogenic teratoma formation [91]. Subsequent studies using rodent models, including a humanized mouse model to reconstitute a human immune system, produced conflicting findings. Whereas some reports [92,93] found that autologous transplants were not immunogenic, others [94] found significant immune response upon autologous transplant of some types of iPSC-derived cells. The autologous immune response may be caused by aberrant antigen expression induced by differentiation in vitro and might be cell type−specific. For example, iPSC-derived smooth muscle cells showed aberrant antigen expression but iPSC-derived retinal pigment cells did not [94]. A more recent study demonstrated that rejection of autologous transplants can occur in rare cases, due to increased mutation burden in the mitochondrial DNA and representation of neoantigen from these mutations [95]. Therefore even for autologous transplantation, preclinical studies should include analysis of potential immune response.

Besides the potential immunological advantages of iPSCs, any cell therapy shares the common challenge of properly delivering the cell product [96]. For most cases, delivery of therapeutic cell types must be targeted to a region of interest. Although intravenous injection is simple and easy, it often results in cell capture and the destruction of delivered cells in the lungs or the liver. Consider, for example, the case of myocardial infarction. A myocardial infarct results from the loss of blood flow to a region of the heart, typically resulting from an occluded vessel. The chronic effects of an infarct are physical remodeling of the heart muscle tissue that hypertrophies as the heart struggles to adapt a loss of function. In this case, restoration of muscle function through cell replacement could stop the remodeling process and provide symptomatic relief. Preclinical studies on rodent and

nonhuman primate myocardial infarction models reported that transplantation of ESC-derived cardiomyocytes can be effective to prevent further deterioration of cardiac function by remuscularization of myocardial infarcts [97]. However, the heart is not a mechanically hospitable environment for cell delivery. Cells must be physically and electrically integrated in an appropriate manner to provide meaningful improvements and avoid potentially harmful side effects in humans.

In other areas of the body, such as the central nervous system (CNS), transplanted cells are less likely to escape into the bloodstream. The relative ease of delivery is in part why most PSC-based therapies have focused on the CNS. Several groups have developed cell replacement therapies to treat macular degeneration, which is caused by the loss of the retinal pigment epithelium (RPE). The first trial to use iPSCs for any disease was led by Dr. Masayo Takahashi in Japan; the first patient was transplanted with autologous iPSC-derived RPE cells, but a decision was made to continue the study using allogeneic (unmatched) iPSC-derived RPE; there is discussion about using an HLA-matched Japanese iPSC bank for further trials [98]. Recently, another research group led by Dr. Kapil Bharti has decided to proceed with clinical trials using autologous RPE cells after determining that in nonhuman primates, allogeneic RPE was rejected by the host's immune system [99,100].

PD is another area of intense interest for a PSC-derived cell therapy. PD results from progressive loss of A9 dopaminergic neurons in the substantia nigra, and by the time the symptoms are diagnosed properly, over 50% of the neurons have already been lost. Methods for producing dopamine neurons from human PSCs have improved over the last few years [54]. When neurons whose activity could be optogenetically controlled were transplanted into a rodent PD model, these neurons could integrate into host circuitry, secrete dopamine, and restore movement control [55]. Clinical trials are planned in multiple countries, including Japan, the United States, and Sweden. A report from a meeting of the four major groups planning such therapy indicated that two groups plan to use hESC-derived dopamine neuron precursors, one plans allogeneic therapy with a single iPSC line, and the fourth plans an autologous iPSC approach [101].

There are now robust methods for the generation of specific cell types from hESCs and iPSCs that work for all cell lines regardless of type or genetic background. There is currently considerable discussion about whether the use of autologous or allogeneic iPSCs is a better choice for transplantation [102]. Allogeneic cells, hESCs and unmatched iPSCs, have been reported to be rejected in nonhuman primate studies [99,103], and human trials using allogeneic cells are all planning immunosuppression for at least a year to prevent graft rejection. The debate about which cells are preferable revolves around the main issues of cost of production of autologous cells and risks associated with immunosuppression for allogeneic therapy.

Other applications for induced pluripotent stem cells

The characteristics of iPSCs, their ability to proliferate without limit and differentiate into multiple cell types inspires other potential uses. One of the least straightforward and most challenging applications for iPSCs is their potential for use in wildlife conservation. One such example is the ongoing effort to save an endangered species, the northern white rhinoceros (NWR), which is on the brink of extinction owing to poaching and civil wars. The two remaining NWRs in the world, two females, cannot carry a pregnancy even if artificial insemination with stored NWR sperm is used. In an effort to save the species, iPSCs were first made from cryopreserved NWR fibroblasts in 2011 [104]. This success raised hopes for applying iPSC technology to differentiate NWR iPSCs into gametes to use for assisted reproduction. In 2015 an international group of researchers met in Vienna in 2015 to develop a multifaceted plan to save the NWR using assisted reproduction technology, including iPSCs [105]. This approach is possible only because of the foresight of researchers who, over the past three decades, preserved dermal fibroblasts from 12 genetically diverse NWR individuals in the Frozen Zoo at the San Diego Zoo Institute for Conservation Research (http://institute.sandiegozoo.org/). Since 2011 fibroblasts from several more NWRs have been reprogrammed; the technical hurdles now lie in producing functional gametes and successfully implanting fertilized embryos into surrogate hosts. It was reported that functional oocytes were generated entirely in vitro from mouse iPSCs, which provides further hope that the same can be done with endangered species iPSCs [106].

Conclusion

For decades, human diseases were modeled in the mouse because of the genetic tools available to alter the mouse genome. Then hESCs began to be used to generate multiple human cell types that could be used for clinical cell therapy and disease modeling. However, hESCs lacked ethnic diversity [107], and although there are hESC lines with genetic mutations, they cannot be linked to the phenotype of a living individual. Human somatic cell reprogramming has revolutionized the fields of stem cell biology, regenerative medicine, and the study of human disease. iPSCs share all of the benefits of hESCs and also have the advantages of genomic diversity and linkage to specific individuals whose medical history is known.

The future of reprogramming is difficult to predict because the technology is developing so quickly. For example, one can imagine moving from the culture dish to in vivo reprogramming. Such an approach could be used to augment tissue-specific stem cells to enhance regeneration; however, improvements are necessary in the control over the delivery of the factors in vivo. Interestingly, a study investigated the short-term expression of the Yamanaka factors in genetically engineered rodents and reported a prolonged life span and decreased recovery time after injury [108], balanced by a higher rate of tumor formation. Studies such as this open the door to potential therapies based on iPSC technology.

A complementary technology that is also evolving at a rapid pace is the CRISPR/Cas9 system and other genetic engineering methods that enable targeted genomic editing. Efficient genome editing of iPSCs can be used to correct mutations, allowing autologous cell therapy for genetic disease. Gene editing can also produce better models for disease, allowing the introduction of specific disease-related variants into iPSCs that can be used to better understand phenotypic expression of mutant and wild-type alleles. The applications of iPSCs are a new tool in 21st-century medicine that will improve our understanding of human disease and enable novel approaches to treat currently untreatable diseases.

List of acronyms and abbreviations

ALS amyotrophic lateral sclerosis
CFTR cystic fibrosis transmembrane conductance regulator
ESCs embryonic stem cells
GWAS genome-wide association study
hESCs human embryonic stem cells iPSCs Induced pluripotent stem cells
MET mesenchymal−epithelial transition
NWR northern white rhinoceros
PD Parkinson's disease
RPE retinal pigment epithelium
SNCA alpha-synuclein
TS Timothy syndrome

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Chapter 26

Neonatal stem cells in tissue engineering

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Introduction

In recent decades, fetal diagnosis and therapy have risen to prominence, and with this rise, so too has regenerative medicine started to develop in areas relevant to the fetus and the newborn. Concerning congenital anomalies, a deficiency of tissues in the patient may prevent anatomical reconstruction, and current treatment strategies involve either using an artificial material or a nonanatomical conduit to surgically repair the defect. This has opened new possibilities for tissue engineering solutions for several congenital anomalies.

There are important considerations to be taken when regarding fetal and neonatal tissue engineering: the availability of fetal/neonatal stem cells, the differences between perinatal and postnatal tissue regarding maturation profiles and manipulation capabilities of the tissue ex vivo, and the conditions appropriate for treatment via a tissue engineering approach. Fetal and neonatal cells may also interact differently when seeded upon certain scaffolds to build specialized tissues.

Specific to scaffolding related to the fetus and newborn, the potential for "cellurization in vivo" is an attractive prospect because of the natural ability of cells to grow and to form new tissues during development.

The first part of this chapter will summarize the current landscape of tissue engineering in the fetus and newborn from both stem cell biology and scaffolding perspectives before moving on to describe some of the experimental and clinical applications of tissue engineering in treating a variety of different congenital anomalies.

Stem cells

The use of stem cells in tissue engineering has revolutionized the landscape of the field; these cells can be maintained in culture to varying degrees and can be manipulated through environmental and genetic modification to fulfill a desired role.

Embryonic stem cells

Embryonic stem (ES) cells, found in the inner cell mass of the blastocyst, are extremely widely potent (differentiating into cell lines from each of the three germ layers). Human ES (hES) cells can be isolated and cultured in vitro, and optimal conditions for proliferation have been developed using fibroblast feeder layers and fibroblast growth factors [1]. Further work has also been done to identify the role of transcription factors to suppress differentiation genes within the hES cell (Oct-4, NANOG, and SOX2) [2].

The limitations to clinical applications for hES revolve around their extensive potency and the acquisition of the cells themselves. Prior to implantation in a host, hES cells require very specific direction for appropriate lineage commitment or risk, forming many varied cell types, and bear the potential for teratoma formation. Clearly, these cells must also be allogeneic in origin; since by such a time as a diagnosis of congenital malformation would be made within the fetus, hES will no longer persist within the developing organism. Until recently, the derivation of ES cells has involved the creation of an embryo solely for ES cell harvest, and subsequent destruction of the embryo, but advances have been made whereby ES cells could be isolated from the developing embryo without disrupting its growth [3–5]. This offers a possibility of preserving autologous ES cells for the developing fetus in the case of IVF during preimplantation manipulation. Clearly, the approach of harvesting ES cells from "expendable" embryos bears several ethical dilemmas that are avoided by the use of stem cells derived from the "patient" fetus itself, as discussed in the following sections.

The concept of somatic cell nuclear transfer (SCNT), or the substitution of a cultured oocyte with a nucleus from a donor cell [6], is an alternative means of generating ES cell lines that are functionally autologous in terms of the cell immunogenicity. This technology rose to fame

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with the widely publicized cloning of Dolly the sheep [7] but has proven to successfully generate blastocyst-derived ES cells, from which tissues may be engineered in vitro. The translational adoption of SCNT has been limited, however, largely owing to the paucity of human oocytes.

Induced pluripotent stem cells

Inducing pluripotency in somatic cells to create induced pluripotent stem cells (iPSC) is a technique developed in the past decade, first by Yamanaka and colleagues in Kyoto [8]; the process involves the inducing factors associated with pluripotency in ES cells (namely, Oct3/4, Sox2, c-Myc, and Klf4), which reverses the commitment to cell lineage in adult cells in what could be dubbed dedifferentiation. The therapeutic potential of iPS is limited at present, since current methods of induction require use of retroviral vectors that are nondiscriminative in their integration sites within the genome, giving concerns of undesired off-target effects, including tumorigenesis. There have been methods described where induction of pluripotency is achieved with nonintegrating plasmids [9], or nonviral RNA vesicles [10] that are thought to be more acceptable; however, these still do not negate the risk of tumor formation.

The creation of pluripotent stem cells from a donor with an established genetic disorder opens the possibility of gene correction, expansion, and subsequent reimplantation and is an exciting prospect in the treatment of several hemoglobinopathies such as beta-thalassemia and sickle cell anemia [11]. With respect to perinatally acquired stem cells, it has been established that these may be easier to reprogram than adult somatic cells, and this may be advantageous for establishing perinatal therapies. In this regard, it has been shown that first and second trimester CD117 + amniotic fluid (AF) stem cell (AFSC) can be converted into iPSC in a transgene-free fashion by an addition of the FDA-approved chemical valproic acid into the culture media [12].

Perinatal stem cells

Postnatal cells are limited in their potential and, even after reprogramming, maintain epigenetic modifications, which may make their application less safe and effective for patients. Perinatal cells acquired during gestation or soon after delivery may overcome these limitations. While, at birth, cord and placenta are certainly valid alternatives, during gestation AF is certainly a very appealing cellular reservoir.

Cells from the umbilical cord, amniotic membrane, and placenta

Different cell types, such as trophoblastic, hematopoietic, epithelial, and mesenchymal stem cells (MSCs) [13], can be isolated from placenta, either during pregnancy or at the time of delivery [14,15]. While it is still unclear if pluripotent stem cells persist during development beyond the first few weeks [16], the placenta has been demonstrated to contain various stem cell niches, which may reflect the different embryonic origin of its parts. Of relevance, placenta has been reported to contain a population of broadly multipotent stem cells that also show the expression of ES cell markers as c-KIT, OCT4, SOX2, SSEA-3, SSEA-4, TRA-160, and TRA-1-81 [17,18]. These cells have a mesodermal phenotype but show a broader differentiation potential, not limited specifically to mesenchymal lineages, but can differentiate into hepatocytes, vascular endothelium, pancreatic, and neuronal cells [19,20]. Another cell type with relevance is a population of human amnion epithelial cells (hAECs), derived from placenta, which has shown therapeutic promise in preclinical models of a bronchopulmonary dysplasia (BPD), a chronic lung disease that mainly affects premature babies who require ventilator support [21]. The first-in-human clinical trial of hAECs in babies with BPD has recently been started in Australia; allogeneic hAECs (1×10^6/kg bodyweight), by intravenous infusion to six premature babies with BPD, have been safely infused, and future randomized clinical trials to assess efficacy in this patient population are currently underway [22].

Umbilical cord blood–derived cells

The umbilical cord has, along with the placenta, shown to be a valuable source of widely potent stem cells, with limited ethical conflicts in their harvest and culture. In 2005 the group of Denner in the United Kingdom demonstrated embryonic characteristics in cells that were obtained from cord blood at the time of delivery, expressing markers familiar in examples of ES cells (Oct-4, SSEA-3, and SSEA-4, among others) [23]. Cord blood–derived cells have been used in literally thousands of published preclinical models, and several hundred clinical trials are currently underway or have completed with uses spanning from the treatment of hematological disorders to the prevention of tissue ischemia in stroke and myocardial infarction [24].

Amniotic fluid stem cells

AF can be collected safely during second trimester routine amniocentesis (at 14–16 weeks gestation), third trimester amnioreduction (at 28 weeks or later), or cesarean section (end of gestation) and the discovery of fetus-derived stem cells within this fluid has opened up many exciting avenues in tissue engineering [25]. The role of the stem cells within the AF are yet to be clearly defined; it is becoming apparent that extracellular vesicles of AFSCs play a key role in many of the therapeutic effects observed by these cells

[26]; however, work remains to be done to identify from where in the fetus these cells originate, how they find their way into the AF and for what purpose.

AFSCs are broadly multipotent; they may commit to lineages of fat, bone, muscle, endothelium, liver, and neural tissues, thus forming cells of any germ layer. They represent approximately 1% of cells within the AF and can be purified from AF by selection of their expressed CD117. Following selection, AFSC can be cultured and refined in vitro without any need of a feeder layer (which is of importance when considering clinical applications since feeders commonly use xenogeneic cells).

Most importantly when considering broadly potent stem cells, AFSCs do not cause cancers when transplanted, neither when reimplanted back to the donor nor when transplanted to other recipients. Within the AF cell population also exist MSCs (AFMSCs). These cells possess only mesodermal potential (they do not display markers of hematopoietic progenitors), have been shown to grow rapidly in cell culture, and have significant potential at generating extracellular matrix (ECM) components [27].

Given that access to AFSCs often already occurs during the diagnostic workup of many antenatally identified anomalies, there is a preexisting means whereby highly potent stem cells can be obtained and manipulated during the remainder of pregnancy in preparation for either a fetal or neonatal repair.

Scaffolding specifics in fetal and neonatal tissue engineering

Engineered scaffolds that can be seeded with cells to form a structural and functional tissue mark an interface between cell biology and materials science. Several important attributes are desirable to successfully fulfill the requisite role of a scaffold: biocompatibility, biodegradability, and mechanical properties that closely align to those of the target tissue. Optimal scaffolds will also feature promoters for cell survival and proliferation. Materials can be broadly, but comprehensively, divided into those that are either synthetic or naturally derived [28].

Synthetic materials

Synthetic scaffolds represent the most immediate clinical prospect within tissue engineering; by their nature they can be reproduced precisely and relatively cheaply and may be developed to display a range of mechanical properties. Within the wider field of clinical surgery, synthetic patches, grafts, and sutures are already commonly used and have an excellent safety profile displaying minimal immunogenicity and a degradation and permeability profile that can be altered to requirement—the latter being particularly important when considering the necessity for neovascularization in large-scale tissue constructs [29–31].

A key feature of synthetic scaffolds must be the ability for cells to integrate into the structure; nonabsorbable materials, such as polytetrafluoroethane (PTFE, marketed commonly as Teflon or Gore-Tex), will cause a foreign body reaction when implanted, and a fibrous capsule is routinely found around implanted patches, currently used for defect closure in a variety of settings. While the structural properties of PTFE allow it to close defects effectively, the inability for cell ingrowth and, importantly, internal angiogenesis preclude it, and other similar materials, from being used in a tissue engineering capacity. The persistence of the material also gives concern for scaffold erosion through nearby structures—as has been described in the adult case report literature with aortic grafts, tracheal, and esophageal stents invading adjacent structures in the mediastinum. In neonatal surgery, Gore-Tex patches are commonly used to close large defects in congenital diaphragmatic hernia (CDH) [32]; however, issues arise from the inability of the patch to grow with the child, including chest wall and spinal deformities [33,34]. Indeed, as a rule, adapting a synthetic scaffold to feature naturally occurring molecules, such as collagen, markedly improves the capacity of the engineered structure to adhere cells within the prospective recipient, allowing for true tissue integration.

Polyglycolic acid (PGA) and poly-L-lactic acid (PLLA) are two prominent synthetic materials with FDA approval for clinical use. PGA has been demonstrated to provide excellent structures on which to culture smooth muscle cells (SMC), degrading completely after 8 weeks, is replaced by SMC-produced ECM proteins [35]. PLLA behaves similarly in vivo and has proved to be a successful scaffold for isolated endothelial cells [36]. Polycaprolactone (PCL) is a further polymeric material with a longer degradation profile than PGA or PLLA (approximately 2 years). As expected, coating PCL with dopamine, collagen, or fibrin increases its cell-adhesive properties, by removing the hydrophobic domains from the exposed surface of the material, and providing receptors for cell adhesins [37–39].

Natural materials

Naturally formed ECM, such as collagen, has the advantage of a structure that lends itself well to cell adhesion and proliferation. Commercially, collagen can be manufactured using recombinant plasmids in yeast or bacteria and can promote differentiation of a variety of different tissues both in vitro and in vivo; importantly, it stimulates internal angiogenesis when seeded with adipose tissue-derived stem cells [40]. Collagen displays low immunogenicity and can be altered in porosity, resorption, and

mechanical properties per requirements. Innovative means of building collagen scaffolds to template have been developed by causing foreign body reaction within the host—forming a so-called biosheet, with purposing of this for patch repair of diaphragmatic and tracheal defects already demonstrated in animal models with encouraging results [41,42]. Since the protein domains across different species are maintained, the immunogenicity of nonhuman collagen is not an issue for clinical application—indeed we see this in clinical practice where porcine and bovine collagen are currently used in repairing and replacing surfaces in a broad range of surgical procedures [43–45].

Similarly, in manufactured matrices of natural components, naturally occurring tissues can be exposed to a variety of enzyme- and detergent-based treatments to achieve total decellularization [46]. This allows the preservation of ECM proteins, and, significantly, the structure can be preserved (albeit some decellularization techniques have proved to be more effective than others at complete preservation). In maintaining the ECM structure, on the microscopic and macroscopic level, the environment for implanted cells or ingrowing is more dependable, and the mechanical and angiogenic properties simply persist, as opposed to having to be engineered to purpose. Once implanted with stem cells, the structure tends toward taking on a histological architecture resembling that of the original tissue.

Decellularized tissues have been utilized for engineering organs in many promising in vivo experiments to date; however, the arrival of clinically relevant technology for use in humans has so far been limited, although excellent results have been reported in the use of acellular dermal matrix in the treatment of burns for many years [47]. Hollow organ engineering is conceivably simpler than achieving the functional capacity of solid tissues such as liver or heart tissue, and our group report successful outcomes in the use of an autologous stem-cell seeded, decellularized tracheal graft as a treatment for congenital tracheal stenosis—with gradual epithelialization and mechanical strength developing over the 2 years following transplantation [48].

Relevance to prenatal therapy

Tissue engineering–based treatment of congenital anomalies is fast becoming a possibility in the antenatal period. Precision with current diagnostics means that a very accurate estimation of the nature and size of a required tissue graft is possible. Furthermore, the marked increase in growth during the final trimester of pregnancy means that fetal application of a tissue graft allows for a far smaller construct to be used—tackling both engineering and clinical issues related to repairing larger structures. As with any fetal intervention, a thorough evaluation of the risks and benefits needs to be performed; many candidate conditions can be treated in the neonate or older infant without any apparent disadvantage, foregoing the ethical and legal dilemmas of fetal intervention and avoiding the risk of premature delivery. However, there are certainly situations whereby a fetal approach may improve outcomes owing to a progressive condition that can be arrested by intervention in utero [49–51], or those where the transition from fetal to neonatal physiology may impact survival such as in the development of pulmonary hypertension in cases of severe CDH [52]. The accessibility of fetal stem cells in the AF may allow for an ex vivo engineering of a bespoke implant before reimplantation into the patient before or after birth. Furthermore, the fetus has several unique considerations as a recipient to a tissue-engineered implant, which will now be detailed more thoroughly.

Immunology

The maturation of the fetal immune system and the phenomenon of tolerance is an important area to explore regarding the fetus' suitability as a graft recipient. The adaptive immune system begins to develop in the eighth gestational week and is thought to be complete by 20 weeks [53,54]. During this process, positive and negative selection of T-cell precursors results in pro-tolerance regulator T cells (T_{reg}) that suppress immune-reactivity to molecules recognized as "self." This concept can be exploited in the transplantation of allogeneic grafts which may then be tolerated without any need for immune modulation. Liechty et al. have described a persistence of allogeneic stem cells in a sheep model after the supposed tolerance threshold, which has led to the hypothesis of "tolerogenic" cells or molecules that may be exploited for cell therapy in the third trimester fetus [55].

Physiology

The placenta and amnion act as the recovery unit for the fetus after an invasive procedure and appear able to autoregulate blood flow to support fetal homeostasis [56,57]. Further technological advances in the development of an ex uterine support system by Flake, Partridge and colleagues in Philadelphia have demonstrated the possibility of an artificial womb where, hypothetically, fetal subjects may recover after major procedures after a deliberate or unplanned premature delivery [58]. There are clear differences in wound healing between the fetus and neonate, with considerably reduced scarring seen related to wounds made in utero [59,60]. This has important implications where invasive procedures are concerned, and developments have gone still further to develop tissue-engineering solutions for the closure of skin wounds in utero [61].

Conditions of interest

Fetal implantation of an engineered construct really only merits consideration under those conditions where the course of the disease may be markedly altered with successful treatment at the earlier stage of development. A staged approach with fetal cell harvest, leading to a planned early neonatal intervention, benefits from the potency of the aforementioned fetal stem cells, while minimizing risk to mother and fetus of a prolonged fetal procedure. We will now explore preclinical models of congenital anomalies with both fetal and neonatal intervention.

Spina bifida

Fetal treatment of spina bifida or meningomyelocele (MMC) has been shown in randomized control trial to benefit patients beyond neonatal repair and has been adopted widely in many centers across the world, including the authors' own. The antenatal closure of the defect avoids the damage to the exposed neural and reverses the development of the Chiari malformation [50,62]. As of yet, the current approach lacks the potential to regenerate the damaged neuronal tissue. A tissue-engineering approach to spina bifida has been considered in a number of animal models, and in addition to defect coverage, there is also clear potential for neural regeneration. A widely adopted approach is to introduce a regenerative scaffold over the exposed spinal cord and fix this in place by closing the overlying skin [63–65]. The use of a tissue-engineered patch with covering sealant to the defect (as opposed to a sutured repair) allows attempts at closure at an earlier gestation (with an empirically smaller defect and reduced extent of nerve injury). Recent results published by Farmer et al. demonstrate a clear benefit of using patch closure with stem cell seeded scaffold in a rodent model of MMC where decellularized ECM scaffolds were used for MMC and showed a clear benefit of those scaffolds that had been seeded with stem cells derived from human placenta [66].

Gastroschisis

Gastroschisis (GS) is also increasingly considered to be amenable to fetal repair. In this ventral abdominal wall defect, the free bowel being exposed to the pro-inflammatory substance of the AF is thought to lead to prolonged intestinal dysmotility that may be lifelong. Transamniotic stem cell injection with AFMSCs has been shown to mitigate bowel damage in both small and larger animal model of GS [67,68]. Experimental models of GS have also been effectively closed with collagen scaffold approaches, demonstrating effective replacement of the scaffold by multiple cellular and ECM components, as well as limiting the inflammatory peel found on the bowel at autopsy [69,70]. The most complex patients with GS will also benefit in the future of the work done around bowel tissue engineering, and there is an argument for AF cell storage for patients diagnosed with complex GS during gestation.

Congenital diaphragmatic hernia

Patients with CDH may benefit from two distinct regenerative medicine approaches, both regarding the diaphragmatic muscle defect itself and the secondary lung hypoplasia—which accounts for the majority of morbidity and mortality related to this condition. Large hernial defects require a patch in order to achieve closure. Besides reported chest wall and spinal deformity, another long-term complication of patch repair in CDH is rehernia-tion. This is felt to be due to the inability of the patch to grow with the child [71,72], which leads to a weakness along the margin of the repair. There is a hypothesis that a cellular patch would allow growth with the individual and thereby reduce the incidence of herniation. This has been demonstrated to be successful by a number of different groups who presented the superiority of a patch derived from AF-originated fibroblasts [73,74]. It is possible that even a decellularized muscle may be superior to a synthetic scaffold. Indeed, diaphragm-derived ECM was able to promote the generation of new blood vessels, boost long-term muscle regeneration, and recover host diaphragmatic function in a mouse model of diaphragmatic hernia [75].

However, the most important challenge to ameliorate long-term survival for patients with CDH is the lung hypoplasia. Most CDH-associated mortality is indeed secondary to pulmonary hypoplasia and subsequent pulmonary hypertension at the transition to neonatal circulatory physiology, leading to requirements for extracorporeal membrane oxygenation, but often terminal cardiorespiratory failure. We have demonstrated an integrative capacity of AFSCs, resulting in marked histological improvement of lung hypoplasia in the nitrofen-exposure murine model of lung injury [76], demonstrating improved tissue architecture, motility, and innervation, holding valuable potential for lung regeneration in not only CDH-related hypoplasia, but also in lung immaturity in general. Moreover, the use of human AFSCs can promote pulmonary development in a rabbit model for CDH [77]. This may, in the future, translate clinically similar to what has recently been reported for BPD [22].

Esophageal atresia

A tissue-engineered esophagus is increasingly the focus of several groups for the treatment of esophageal atresia

[78,79]. We have published preliminary data on a murine decellularized model seeded with stem cells, demonstrating effective seeding of mesangioblasts and neural crest cells within a natural scaffold [80]. Other groups describe utilizing the aforementioned "biosheet" technique successfully for patch esophagoplasty, demonstrating an effective potential tissue-engineering solution to a condition often managed presently with interposed grafts of nonspecialized intestine [81]. Fetal tissue, such as decellularized human amniotic membrane, has also been adopted for esophageal tissue engineering; myoblasts and oral epithelial cells were seeded, respectively, on acellular porcine ileal submucosa and decellularized human amniotic membrane, and these were used in combination for the circumferential replacement of the cervical esophagus in pigs [82]. The same group describes a similar experiment 2 years later utilizing autologous MSCs seeded on an acellular matrix, demonstrating an essential role for the MSC in promoting ingrowth of the epithelium [83].

Congenital heart disease

Fetal echocardiography now allows for detailed diagnosis of the most clinically significant congenital heart diseases. For many the transition of the circulation at the time of the closure of the ductus arteriosus (DA) leads to a circulatory collapse if untreated. Current management typically involves prostaglandin infusion to maintain patency of the DA in order to plan and deliver a surgical intervention, such as a patched repair or conduit construct using one of the aforementioned materials [84].

Experimental evidence suggests that AFSCs may express cardiac myocyte markers and develop electromechanical connections. Furthermore, they provide a source of functional endothelial cells from which coronary vessels and endocardium may originate [85]. In addition, Chorionic Villus Sampling (CVS)-harvested MSCs have been developed into living heart valve tissues after coculture with cord-blood endothelial progenitors [86]. With the precision of fetal echocardiography in the modern era, we are fast approaching an era where fetal tissues may provide materials for the construct of a patient-derived graft amenable to implantation in postnatal life (Fig. 26.1).

Congenital airway anomalies

Congenital high airway obstruction syndrome, or laryngotracheal agenesis, is known to many fetal medicine specialists who provide the rationale for tracheal occlusion in the treatment of lung hypoplasia in CDH. This condition is almost uniformly fatal around the time of birth, requiring an EXIT (ex utero intrapartum) procedure or emergency neonatal tracheostomy [87]. Airway reconstruction is the only treatment option, and approaching this anomaly with a similar approach to that described previously,

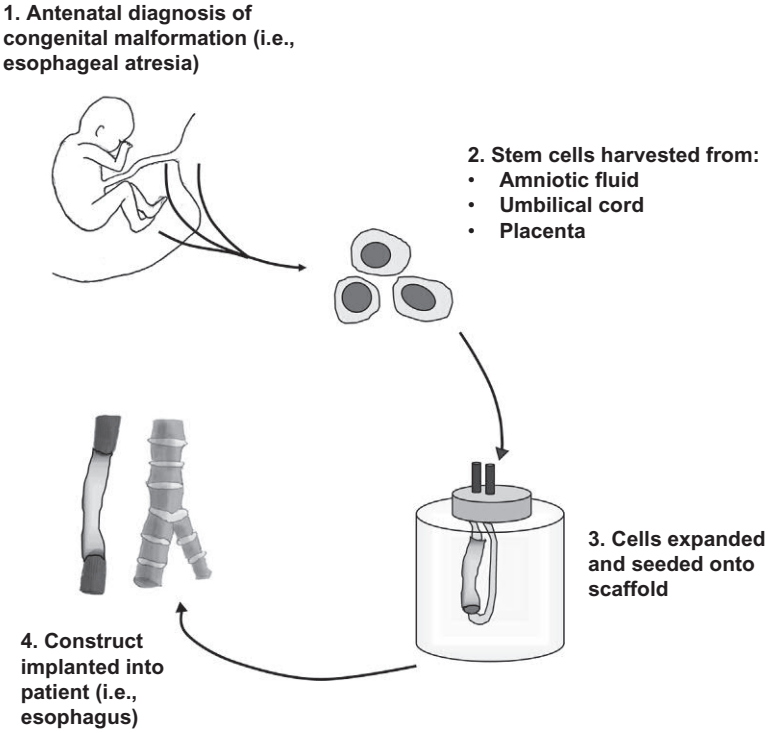

FIGURE 26.1 With accurate antenatal diagnostics, a need for a bespoke organ replacement can be defined. Cells can be harvested from a perinatal source and implanted into the newborn.

with a scaffold seeded with autologous cells obtained during gestation, may prove to be a potential therapy option in this devastating condition [48,88].

Bladder

Bladder exstrophy, with or without an associated cloacal anomaly, is a further area of active research within tissue engineering. Current approaches to reconstruction are limited in terms of achieving the required contractility or elasticity for a satisfactory function of the neo-bladder, and a tissue-engineered solution is a potential means of achieving an improved outcome in this regard. Indeed, Atala et al. reported in 2006 the successful use of collagen or collagen−PGA scaffolds, seeded with autologous urothelial and SMC successfully in human patients with spina bifida who were suffering from poor compliance or high intravesical pressure [89].

Bone and bone marrow

Existing reconstruction of skeletal defects in humans utilizes free bone grafting, various scaffolds with or without seeded bone marrow−derived adult stem cells, and the use of adjuncts such as bone-morphogenic protein [90]. AFMSCs and AFSCs have been demonstrated to produce bone mineral matrix with prolonged mineral production. Both have been utilized to create bone grafts used to surgically correct thoracic and craniofacial defects in animal models [91−93]. Moreover, there is a real possibility of contributing to the treatment of some devastating diseases that can be diagnosed prenatally such as osteogenesis imperfecta (OI). OI results from a defect in the synthesis of type-1 collagen, most commonly (>90% cases) a mutation in the subunit COL1A1 or COL1A2. Severe cases of OI present with prenatal fractures and existing therapy aim to palliate symptoms and decrease acquired deformity. Prenatal transplantation of allogeneic MSCs in OI appears safe, and a clinical trial (phase I/II) is taking place currently in the form of the BOOSTB4 trial [51]. Preclinical benefits have also been shown with AFMSCs. Intraperitoneal injection of human (AFMSCs) into a mouse model of OI reduced fracture susceptibility, increased bone strength, improved bone quality and micro-architecture, normalized bone remodeling, and reduced TNFα and TGFβ signaling [94].

One of the most promising applications of AFSCs is in the ability these cells display to engraft and reconstitute the hematopoietic system [95]. In utero stem cell transplant has long been used in isolated cases of severe immune-deficiencies in humans with encouraging, clinically relevant levels of engraftment [49,96]. It has also been noted that the tolerogenic properties of AFSC seem to allow transplantation at a later gestational age than would be predicted in terms of the onset of fetal tolerance during immune maturation [55].

Conclusion

Progress made in the field of regenerative medicine has direct implication in the development of innovative prenatal and neonatal treatments. Following the last two decades of scientific work, we are now witnessing the initial translation of this into early clinical trials. Both fetal and neonatal interventions with bespoke tissue or whole organ replacement are rapidly becoming a possibility, and the next decade of scientific research promises to deliver many exciting breakthroughs in the care for these patients.

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Chapter 27

Embryonic stem cells as a cell source for tissue engineering

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Introduction

It has been estimated that approximately 3000 people die every day in the United States from diseases that could have been treated with stem cell–derived tissues [1]. Given the therapeutic potential and growing public awareness of stem cells to treat disease, it is not surprising that embryonic stem cell (ESC) research has been rapidly expanding since mouse ESCs (mESCs) were first isolated in 1981 [2,3] followed by the isolation of human ESCs (hESCs) in 1998 [4,5] from the inner cell mass (ICM) of human blastocysts (Fig. 27.1). As the development of mouse and human embryos is different, ESCs form these two can also have disparities [7]. Adult stem cells have been used clinically since the 1960s for therapies such as in bone marrow transplantation, and these cells hold great therapeutic promise. ESCs also offer major benefits, including their ease of isolation, ability to propagate rapidly without differentiation, and—most significantly—their potential to form all cell types in the body. In addition, ESCs are an attractive cell source for the study of developmental biology [8] screening of drugs/toxins [9] and the development of therapeutic agents to aid in tissue or organ replacement therapies [10]. Regarding the latter application, which is the focus of this chapter, ESCs have the potential to exhibit a considerable impact on the field of tissue engineering, where current treatments for large tissue defects involve graft procedures that have severe limitations. Specifically, many patients with end-stage organ disease are unable to yield sufficient cells for expansion and transplantation [11]. In addition, there exists an inadequate supply of harvestable tissues for grafting, and that which is available has associated risks, such as donor site morbidity, infection, disease transmission, and immune rejection [12].

Tissue-engineering-based therapies may provide a possible solution to alleviate the current shortage of organs available for transplantation. Expectations for the potential of stem cells have increased even more after the revolutionary generation of induced pluripotent stem cells (iPSCs) that profoundly modified the principles of cell fate and plasticity, and at the same time, may represent a novel remarkably important tool for cell-based therapy [13]. iPSCs were originally generated by the introduction of four transcription factors (Oct3/4, Sox2, Klf4, and cMyc) in a somatic committed cell, the fibroblast, converting it to a pluripotent ESC-like state [14]. This work

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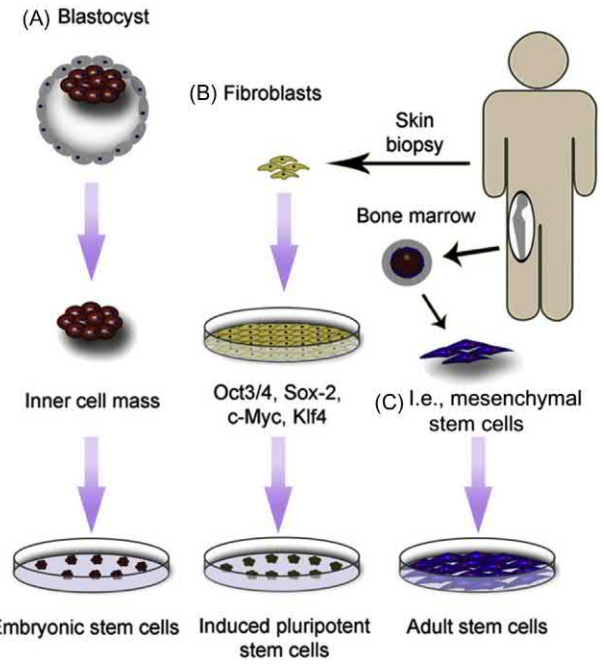

FIGURE 27.1 Schematic diagram of the derivation of stem cells.
(A) Derivation of embryonic stem cells from the inner mass of the blastocysts and differentiation to different cell types; (B) generation of induced pluripotent stem cells from somatic cells overexpressing Oct3/4, Sox2, c-Myc, and Klf4; and (C) formation of ASCs during ontogeny (e.g., bone marrow mesenchymal stem cells). *ASCs*, Adult stem cells. *Adapted from Martino S, D'Angelo F, Armentano I, Kenny JM, Orlacchio A. Stem cell-biomaterial interactions for regenerative medicine. Biotechnol Adv 2012;30(1):338–51 [6].*

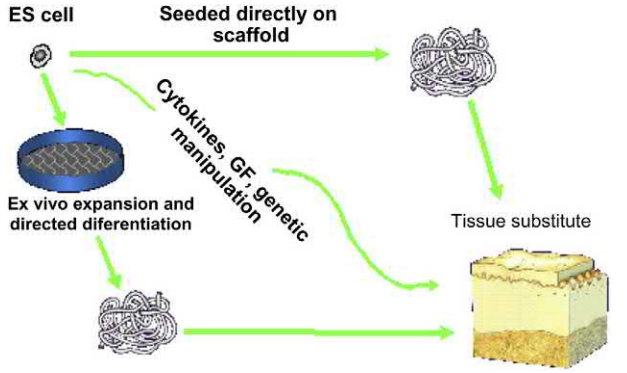

FIGURE 27.2 Approaches for using ES cells for scaffold-based tissue engineering.
ES cells can be used in tissue-engineering constructs in a variety of methods. ES cells can be expanded in culture and then seeded directly onto scaffold where they are allowed to differentiate. Alternatively, stem cells can be directed to differentiate into various tissues and enriched for desired cells prior to seeding the cells onto scaffolds. *ES*, Embryonic stem.

gave rise to a completely new field that is not covered in this chapter, and the reader is directed to a number of excellent papers and reviews on this topic.

Tissue engineering has been defined as an interdisciplinary field that applies the principles of engineering, materials science, and life sciences toward the development of biologic substitutes that restore, maintain, or improve tissue function [15]. Thus tissue engineering may provide therapeutic alternatives for the treatment of organ failure or tissue defects that are acquired congenitally or produced by cancer, trauma, infection, inflammation, or surgery. Tissue-engineered products would provide a lifelong therapy and may greatly reduce the hospitalization and health-care costs associated with drug therapy while simultaneously enhancing the patients' quality of life.

A central part of such strategy is the cell source to be used, and the methods whereby sufficient numbers of viable differentiated cells can be obtained. ESCs represent a powerful source of cells capable of multilineage differentiation because they can potentially provide a renewable source of cells for transplantation. ESC-derived cells can be used directly as cellular replacement parts or in combination with other materials (typically in the form of scaffolds, Fig. 27.2). Despite this promise, the application of ESCs in tissue engineering faces numerous challenges, including appropriately differentiating the cells to the desired lineage in a controlled and homogenous fashion and avoiding implantation of undifferentiated ESCs which can potentially form teratomas. With advances made in molecular imaging, this can possibly be tracked and detected [16]. Research has also been looking into blocking teratoma formation through targeting antiapoptotic genes [17]. Currently, ESC-based tissue-engineering research is focused on elucidating soluble and immobilized cues and respective signaling mechanisms that direct cell fate, on characterization and isolation of differentiated progeny, and on establishing protocols to improve the expansion and homogeneity of differentiated cells [18,19].

This chapter discusses key concepts and approaches for

1. the propagation of undifferentiated ESCs,
2. the directed differentiation into tissue-specific cells,
3. the isolation of progenitor and differentiated phenotypes,
4. the transplantation of progenitor and differentiated cells, and
5. the remaining challenges for translating ESC-based tissue-engineering research into the clinical therapies.

Whenever possible, approaches using hESCs are reported.

Maintenance of embryonic stem cells

The self-renewal of ESCs is a prerequisite for generating a therapeutically viable number of cells. Over the past few years, much insight has been gained into the

self-renewal of ESCs. Both mESCs and hESCs were first derived and maintained in culture using mouse embryonic fibroblast (MEF) feeder layers and media containing serum. Considerable behavioral, morphological, and biochemical differences have been observed between mESCs and hESCs, and the research of animal ESCs is not easily translated to hESCs [5,20,21]. For example, mESCs form tight, rounded clumps, whereas hESCs form flatter, looser colonies, grow more slowly and demand more strict culture conditions to maintain their normal morphology and genetic integrity. Unlike mESCs, which can be maintained in an undifferentiated state in the presence of leukemia inhibitory factor (LIF), addition of LIF fails to inhibit spontaneous differentiation of hESC [22]. Although both mESCs and hESCs express common transcription factors of "stemness," such as Nanog, Oct4, and alkaline phosphatase, in the human system, undifferentiated ESCs express stage-specific embryonic antigen-3 (SSEA-3) and SSEA-4, and SSEA-1 is only expressed upon differentiation, whereas the opposite expression is observed in the mouse system. Due to these differences, efforts in hESC research focus on understanding the molecular mechanisms of hESC self-renewal. For example, recently, it has been found that that Bach1 plays a key role in self-renewal, pluripotency, and lineage specification in hESCs by stabilizing pluripotency factors [23].

Mouse and human iPSCs have marker expression profile, and biological properties very similar to the mouse and hESCs, respectively, and this is a general proof of principle of their real pluripotent state. However, after initial studies, data are emerging that iPSCs are actually not identical to ESCs [24], and a growing body of evidence indicates that their pattern of gene expression differ [25] and the epigenetic memory of the original cell type reprogrammed is at least partially maintained (reviewed in Ref. [26]). This may result in a more limited level of pluripotency in terms of spectrum of differentiation if compared to ESCs, but not necessarily a more limited spectrum of possibility of tissue differentiation for transplantation purposes if the original cell type is chosen accordingly [27].

Therapeutic applications of stem cells require the use of moderate to large numbers of cells, hence requiring methods amenable to scale up. Therefore xenogeneic cell sources have also been considered. Using cultures of hESCs on human feeder cells, it was found that human fetal muscle fibroblasts, human fetal skin fibroblasts, and adult fallopian tubal epithelial cells supported the pluripotency of hESC culture in vitro [28]. The same group derived and established a hESC line on human fetal muscle fibroblasts in entirely animal-cell free conditions [28]. Since then different fetal and adult cells have been examined and shown to support the continuous growth of hESCs [29–32]. However, the use of hESCs for therapeutic application requires the use of defined culture media and controlled cell derivation, maintenance, and scale-up procedures. To overcome these obstacles, combinations of factors that influence the self-renewal of hESCs have been investigated, including soluble factors, extracellular matrix (ECM), cell–cell interactions, and mechanical forces.

Significant attempts have been made to identify culture conditions and media components which can regulate hESCs self-renewal. Growth factors in culture media can bind the cell surface receptors to promote self-renewal. These soluble factors include basic fibroblast growth factor (bFGF) [33,34], transforming growth factor β1 (TGFβ1)/activin A/nodal ligands [35,36], insulin-like growth factors (IGFs) [34,37], Wnt ligands [38,39], and glycogen synthase kinase-3 (GSK-3) inhibitors [40]. In one study, it was shown that hESCs can be expanded on human fibronectin using a medium supplemented with bFGF and TGFβ1 [41]. Noggin, an antagonist of bone morphogenetic protein (BMP), was found to be critical in preventing the differentiation of hESCs in culture. The combination of Noggin and bFGF was sufficient to maintain the proliferation of undifferentiated hESCs [42].

It has also been demonstrated that Wnt ligands affect ESC self-renewal and differentiation. For example, spontaneous differentiation of MEFs is inhibited by the addition of Wnt1 to culture media [38]. In addition, hESC differentiation is induced by using Wnt3 [22]; however, hESC self-renewal perturbs by the activation of canonical Wnt/β-catenin pathway through the expression of stabilized β-catenin [43]. Furthermore, hESCs maintained in media containing high concentrations of bFGF (24–36 ng/mL), alone or in combination with other factors, show characteristics similar to those in cultures maintained with feeder cell-conditioned medium [44,45].

The derivation of hESCs has also been achieved with minimal exposure to animal-derived material, using serum replacement and human foreskin fibroblasts as feeder cells [46], instead of the feeder cell layer [47], providing well-defined culture conditions [48]. Research is currently underway to determine how these conditions maintain cell integrity over long-term culture. For example, mTeSR hESC culture medium, which contains TGFβ1, lithium chloride (LiCl), bFGF, pipecolic acid, and gamma-aminobutyric acid, supports long-term self-renewal of feeder-independent hESC cell culture [48]. In addition to growth factors, lipid molecules such as sphingosine-1-phosphate [49–51], albumin [52], and synthetic lipid carriers [53] have been shown to regulate the self-renewal and prevent differentiation of hESCs. Although growth factor and media compositions can control hESC self-renewal, challenges, including maintenance of pluripotency, and production of biologically and functionally identical cells, still remain.

In addition to soluble factors, a defined ECM or biomaterial may be required for maintaining the hESC

self-renewal ability. Various biomaterials, such as Matrigel [54], human fibronectin [41], human vitronectin [55], collagen I [56], complex humanized matrices [48], hyaluronic acid hydrogels [57], or calcium alginate hydrogel [58], have been used as a structural support for hESC self-renewal. For example, Xu et al. showed that hESCs can be maintained on Matrigel or laminin and MEF-conditioned media [59]. These conditions support the growth of pluripotent cells, maintain normal karyotypes, preserve their proliferation and high telomerase activity, and differentiation into derivatives of all three germ layers, both in vitro and in vivo. In an attempt to find ideal ECM components or biomaterials for in vitro feeder-cell-free culture of hESCs, Hakala et al. compared various biomaterials, including ECM proteins (i.e., collagen IV, vitronectin, fibronectin, and laminin), human and animal sera matrices, and Matrigel in combination with a variety of unmodified or modified culture media. Matrigel in combination with defined mTeSR1 culture medium was found to be superior matrix for hESC culture compared to other biomaterials used in this study [54]. Similarly, in a combinatorial study, Brafman et al. developed a high-throughput technology, an arrayed cellular microenvironment, to assess the self-renewal of hESCs cultured on different ECMs in media comprising different growth factors. Long-term self-renewal of hESCs was obtained on a biomaterial consisting of collagen IV, fibronectin, collagen I, and laminin in defined StemPro media and MEF-conditioned media [60].

Self-renewal and differentiation of ESCs can be also regulated through intercellular interactions [60–63] and mechanical forces [64–66]. Cell–cell interactions and the formation of ESC colonies affect the self-renewal and spontaneous differentiation of ESCs. It has been shown that the size and shape of colonies play an important role in controlling ESC expansion [63,67]. Various microfabrication technologies have been employed to control ESC shape and size, such as micropatterning of substrate with ECMs [67] to confine colony formation to patterns, or formation of hESCs colonies in three-dimensional (3D) microwells [61,62]. Another important factor in hESC self-renewal is the application of mechanical forces (e.g., cyclic biaxial strain [64,65] or shear stress [66]) to the cells. Although the physiological effects of mechanical forces on self-renewal and proliferation of ESCs remain unknown, it has been shown that these forces can regulate cellular differentiation. For example, fluid flow–induced shear stress has been demonstrated to enhance the elongation and spreading of undifferentiated hESCs and induce vascular differentiation of hESC at higher shear stress [66].

Large-scale production of hESCs is critical for tissue-engineering applications, which require large numbers of cells. It is generally accepted that "classical" laboratory culturing methods are not suitable for the large-scale production of ESCs for therapeutic applications, and new culture systems are needed. Although two-dimensional (2D) methods such as the high density cultures of ESCs have been developed by combining automated feeding and culture methods, 3D culture may be a more suitable technology for large-scale expansion of ESC production.

At present, the aggregation of multiple ESCs is necessary to initiate embryoid body (EB) formation. The formation of large cellular aggregation may prevent nutrient and growth factor diffusion as well as metabolic waste removal from the aggregates in suspension cultures in large-scale systems. A small number of methods have been developed for the differentiation of mESCs in controlled cultures. Hanging drops and methylcellulose cultures have been shown to be somewhat efficient in preventing the agglomeration of EBs, but their complex nature makes their upscaling a rather difficult task.

A much simpler process in spinner flasks resulted in the formation of large cell clumps within a few days, indicative of significant cell aggregation in the cultures [68]. Compared to static culture system, spinner flasks enhance homogenous expansion of human EBs (hEBs) and can be easily scaled up to 10,000 L bioreactor tanks [69]. In one study, it was demonstrated that the growth rate of hEB is higher when cultured in stirred vessels than in other culture systems (e.g., static culture and rotary cell culture system) [70]. However, an increase of the culture medium stirring rate to avoid agglomeration within the stirred vessels resulted in massive hydrodynamic damage to the cells due to the extensive mixing in the vessels. Therefore in order to establish a scalable process for the development of EBs, there is a need for dynamic cultivation under controlled mixing conditions. One approach used a static system for an initial aggregation period of 4 days, followed by a period in dynamic culture in spinner flasks, to successfully achieve the bulk production of cardiomyocytes from differentiating mESCs [71].

In addition to suspension cultures using hEBs, stirred vessels can be also used for the scale-up expansion of undifferentiated hESCs through the combination of a microcarrier with the stirred culture systems. Various microcarriers such as polystyrene [72], collagen-coated dextran [73], and Matrigel-coated cellulose [74] have been used to promote hESC expansion in spinner flasks. In addition to hESC expansion, this combination of microcarriers and stirred culture systems has been used for directing hESC differentiation to definitive endoderm [75] and cryopreservation and recovery of undifferentiated hESCs adhered to microcarriers [76].

Another dynamic approach that was highly effective for hESCs is to generate and culture EBs within rotating cell culture systems [77]. These bioreactors provide

exceptionally supportive flow environments for the cultivation of hESCs, with minimal hydrodynamic damage to incipient EBs, reduced EB fusion, and agglomeration, and they allow the uniform growth and differentiation of EBs in three dimensions, as they oscillate and rotate evenly. hESCs cultured within these systems formed aggregates after 12 hours that were smaller and more uniform in size and evenly rounded due to minimal agglomeration; the yield of EBs was three times higher than that measured for static cultures. Also, dynamically formed EBs exhibited steady and progressive differentiation, with cyst formation and elaboration of complex structures such as neuroepithelial tubes, blood vessels, and glands [77].

Different rotary cell culture systems, including slow turning lateral vessel (STLV) and high-aspect rotating vessel (HARV), have been used to promote the efficiency of EB formation and differentiation of stem cells [77–80]. Generally, STLV systems are preferable to HARV for the EB aggregate formation and differentiation. It has been shown that the HARV system can lead to significant aggregation with large necrotic areas at the center and differentiations at the peripheries of aggregates. The aggregation rate of hESCs can be controlled by using the STLV system, which results in the formation of small-size hEBs [69,77].

To further enhance the large-scale differentiation of hEBs, a perfused STLV system was combined with a dialysis chamber to allow the diffusion of media as well as removal of waste products from the bioreactor [81]. Compared to static cultures, uniform growth and differentiation of hEBs to neural lineage was promoted when the combined rotary cell culture system/dialysis chamber was used [81]. Although rotary cell culture systems provide low-shear environments for hESCs cultivation and differentiation, they can only be scaled up to volumes of 5–500 mL, which is much lower than the scalability of stirred culture systems. These technologies have demonstrated the potential of engineering for the development of scalable technologies to expand ESC provision for research and therapies.

Directed differentiation

In cell culture, hESCs can spontaneously differentiate into cells of the three germ layers [82]. Perhaps, the biggest challenge in the clinical use of ESCs is the lack of knowledge of how to predictably direct their differentiation. For example, although ESCs can generate cells of hematopoietic, endothelial, cardiac, neural, osteogenic, hepatic, and pancreatic tissues, it has been very difficult to achieve directed differentiation into these tissues. The lack of homogeneous differentiation may be attributed to the intrinsic property of ESCs of differentiating stochastically in the absence of proper temporal and spatial signals from the surrounding microenvironment. Various techniques have been employed to control the differentiation of hESCs and to isolate a specific germ layer for tissue regeneration applications. The limitation of current techniques used for controlled differentiation is the low transformation efficiency, which results in a cell population containing ectoderm and mesoderm germ layers. The segregation of these germ layers can be achieved by using appropriate differentiation protocols. In this section, we describe some of the current approaches used to direct the differentiation of ESCs and give examples of their use.

Genetic reprogramming

To enable the production and propagation of target cell type populations, specific gene(s) are introduced into hESCs. Different techniques for knocking-in and knock-ingout genes into hESCs have already been established. For instance, chemical reagents or electroporation can be used for the transfection of undifferentiated hESCs with specific plasmids. Electroporation has been demonstrated to be useful for the generation of homologous recombination events [83]. Another technique includes the use of self-inactivated lentiviruses for the introduction of transgenes into hESCs, which was shown to be efficient with sustained expression in undifferentiated hESCs as well as in hESCs, which undergoes differentiation [84,85]. However, both the undifferentiated and differentiated hESCs were successfully infected by using adenoviral and adeno-associated viral vectors [86]. Another approach, which uses genetic manipulation, is the introduction of suicidal genes, which permits the ablation of the cells if necessary [87]. Using this approach, hESCs were transfected to express the herpes simplex virus thymidine kinase gene [88].

Genetic techniques involve both positive and negative regulators. The positive regulators include the constitutive or controlled expression of transcription factors that have been shown to derive the differentiation into particular tissues. For example, the overexpression of the Nurr transcription factor has been shown to increase the frequency of ESCs that differentiate into functional neural cells [89] and the use of mir-375 to enhance the differentiation into insulin-producing cells [90]. Alternatively, the negative regulators can be incorporated to induce the apoptosis of cells that differentiate to varying pathways. For example, neomycin selection and suicide genes that are activated by certain transcription factors can be used [91]. Recently, the use of a Forkhead box O1 (FoxO1) inhibitor (Lentiviral silencing) was shown to increase the differentiation of ESC-derived pancreatic progenitors into insulin-producing cells and increased insulin production following stimulation by glucose [92]. FoxO1 has an important

role in metabolic regulation and is profoundly expressed in insulin-responsive tissues [93].

In a study, Zoldan et al. developed a 3D siRNA delivery system using lipid-like materials, lipidoids, for the efficient transfection of hESCs. This system was used to direct the differentiation of hESCs to a specific lineage by knockingdown kinase insert domain receptor to prevent the differentiation of endoderm layer, leading the separation of this germ layer from mesoderm and ectoderm [94]. The developed 3D RNA delivery technique was shown to be preferable over a 2D environment for directing hESC differentiation, in which the transfection reagents are added to the media used for in vitro culture of hESCs-seeded 2D substrates. Clearly, all these techniques would benefit from a deeper understanding of inner workings of transient cells and knowledge of the differentiation pathways and lineages. Recently, the use of a synthetic network to program transcription factors was shown to allow the differentiation of ESCs into insulin-producing beta cells by Ref. [95].

Further analysis of the stem cell and progenitor hierarchy through high-throughput analysis of gene and protein profiles should accelerate this process. Despite the power of these approaches, one potential concern is that the genetic modifications may make the cells unsuitable for transplantation.

Microenvironmental cues

Another approach to directing ESC differentiation is through the use of microenvironmental cues that are important in regulating adult and ESC fate. During development, cells of the ICM are exposed to a series of tightly regulated microenvironmental signals. However, in tissue culture the complex expression patterns and spatial orientation of these signals can be lost. Currently, ESCs are grown in their primitive state as aggregated colonies of cells. To stimulate differentiation, two main methods have been examined. In one method, differentiated cells are derived from EBs. The formation of EBs can be accomplished by using either single ESC cell suspensions or from cell aggregates. EBs have been found to mimic the structure of the developing embryo and recapitulate different stages seen in its differentiation. In addition, clonally derived EBs can be used to locate and isolate tissue-specific progenitors. EBs initiate many developmental processes and help the differentiation of cells into all three germ layers. EBs are generally formed through suspension, encapsulation, and hanging drop methods [96]. More recently, magnetic forces were used to form EBs from iron oxide particle-laden stem cells [97].

In general, the differentiation of ESCs in EBs produces a wider spectrum of cell types, due to the EBs' ability to better mimic the temporal pattern of cell differentiation seen in the embryo. In some applications, combined use of EBs and adherent cultures has resulted in better cell yields. For example, to induce ESC differentiation to cardiomyocytes, EB formation in suspension cultures followed by differentiation in adhesion cultures has been shown to optimize the percentage of cells that give rise to cardiomyocytes [98,99]. Similarly, the production of hepatocytes has been shown to be induced by first culturing the cell in EBs and then in 2D cultures [100].

Neural progenitor cells were isolated from hESCs that showed positive immunoreactivity to neuron-specific antigens, responded to neurotransmitter application, and presented voltage-dependent channels in the cell membrane [101–104]. Various differentiation approaches, including adherent culture or EB suspension culture, have been used to direct the hESC differentiation to the neural lineage [105].

To promote neural differentiation, different soluble factors such as BMP-inhibitors, retinoic acid (RA), and other supplements (e.g., N2, B27, and ITS) are added to the media in adherent culture methods. In EB suspension culture systems, neural induction factors should be added during differentiation to induce neural differentiation of hESCs. The differentiated cells are then cultivated on adherent culture to allow for the neural cell growth. In both approaches the morphological characteristics of the neural progenitors can be maintained, and expression of NP-markers in the medium supplemented with FGF2 and B27 [105]. Highly enriched cultures of neural progenitor cells were isolated from hESCs and grafted into the stratum of rats with the Parkinson's disease [106]. The grafted cells differentiated in vivo into dopaminergic neurons and corrected partially behavioral deficits in the transplanted animals. A subsequent study showed that hESCs implanted in the brain ventricles of embryonic mice can differentiate into functional neural lineages and generate mature, active human neurons that successfully integrate into the adult mouse forebrain [107].

Oligodendrocytes and their progenitors were also isolated in high yield from hESCs [108]. Transplantation of these cells into animal models of dysmyelination resulted in integration, differentiation into oligodendrocytes, and compact myelin formation, demonstrating that these cells displayed a functional phenotype. In addition to in vivo differentiation of hESCs to neural lineages, ESCs can be combined with a biomaterial to induce the in vitro differentiation of ESCs to specific neural lineages in the presence of differentiation-inducing agents. For example, electrospun fibrous scaffolds not only enhanced the differentiation of mouse ESCs into specific neural lineages such as neurons, oligodendrocytes, and astrocytes but also supported the neurite outgrowth [109]. In recent years the use of carbon nanotubes (CNTs) for the neuron

differentiation from hESCs and neural growth has been also explored [110,111]. It has been shown that 2D scaffolds composed of poly(acrylic acid)-grafted CNT thin films promoted hESCs' neuron differentiation efficiency as well as protein adsorption and cell attachment compared to poly(acrylic acid) scaffolds without CNTs [110].

The differentiation of hESCs to neural lineages is induced by supplementation of the culture medium with relevant biochemical agents. Recently, it has been demonstrated that nanopatterning of the substrate can effectively control hESC differentiation to neural lineages in the absence of any biological and biochemical agents. In one study an ultraviolet-assisted capillary force lithography technique was developed to generate 350 nm pattern arrays using polyurethane acrylate [112]. The hESCs seeded on these patterns differentiated to neuronal lineage after 5 days of culture without the addition of differentiation-inducing agents [112]. It was also found that geometry and isotropy influence the efficiency of ESC differentiation and neural differentiation [113]. It was found that with the use of 250 nm topographies is more prone to neural differentiation [113]. Tall nanopillar configuration after chemical stimulation was found to be more efficient for ectodermal differentiation of ESCs [114]. Using gold nanoparticles, it was demonstrated that ESCs can sense topographies having size of less than 5 nm [115].

ESCs have been shown to give rise to functional vascular tissue. Three different strategies have been employed to induce vascular differentiation of ESCs:

1. EB formation
2. Coculture with fibroblast feeder layers or target cells
3. 2D monolayer culture of ESCs in defined chemical conditions combined with differentiation stimuli [116] (Fig. 27.3).

Spontaneous differentiation of ESCs to EB aggregates in a medium supplemented with cytokines has been shown to promote their differentiation to smooth muscle (SM) cells, pericytes, and endothelial cells [117,118]. One limitation of this strategy is that the ESCs differentiate to a heterogeneous cell population composed of vascular cells and other cell types from different embryonic germ layers. Various approaches have been employed to improve the efficiency of EB protocols to promote vascular cell differentiation, such as addition of vascular endothelial growth factor (VEGF)-A [119] or BMP4 [120], to culture media and using magnetic-activated cell sorting or fluorescence-activated cell sorting (FACS) [121].

Another approach to directing the vascular differentiation of hESCs is the use of coculture systems, where undifferentiated ESCs are seeded onto mouse fibroblast feeder layers such as stromal cells [122], MEF [123], or mouse endothelial cells [124] to enhance vascular differentiation. Alternatively, 2D monolayer culture on Matrigel [125], collagen IV [126], and fibronectin [127] combined with differentiation stimuli (e.g., addition of GFs/cytokines [125] or RA [128], mechanical stimulation [129], and hypoxia [130]) has been used for vascular hESC differentiation. Although these strategies increase the differentiation efficiency, isolation of progenitor cells expressing markers, [e.g., CD34, stem cell antigen (Sca)-1, or Flk1] during ESC differentiation is a crucial requirement for deriving homogenous vascular cell populations [125].

Early vascular progenitor cells isolated from differentiating mESCs were shown to give rise to all three blood and vessel cell types: hematopoietic, endothelial, and SM cells [131]. Once injected into chick embryos, these vascular progenitors differentiated into endothelial and mural cells and contributed to the vascular development. hESCs can also be differentiated into endothelial cells by using platelet endothelial cell adhesion molecule-1 antibodies [132]. In vivo, when transplanted into immunodeficient mice, these cells appeared to form microvessels.

Furthermore, it has been shown that monkey ESCs can give rise to endothelial cells when the embryonic cells were exposed to a medium containing combinations of growth factors. The isolated cells were able to form vascular-like networks when implanted in vivo [133]. Endothelial progenitor cells have been isolated from hESCs which presented hematopoietic [134] or SM cells competency. hESCs have been reported to differentiate into hematopoietic precursor cells when cocultured with bone marrow and endothelial cell lines [135]. When these precursor cells are cultured on semisolid media with hematopoietic growth factors, they form characteristic myeloid, erythroid, and megakaryocyte colonies.

Cardiomyocytes have been isolated from hESCs for the treatment of cardiac diseases. The most common approach to induce in vitro differentiation of hESCs to cardiomyocytes is the formation of EB aggregates followed by few days postplating on a 2D substrate to obtain cells with cardiomyocyte characteristics [136]. Cardiomyocyte differentiation of hESCs can be also induced by coculturing undifferentiated hESCs with a mouse visceral endoderm-like cell line (END-2) [137]. Cardiomyocytes isolated from hESCs expressed sarcomeric marker proteins, chronotropic responses, and ion channel expression [137]. One week after culturing in differentiation conditions, beating cells were observed and continued to increase in number while retaining their contractility for 70 days [138]. The beating cells expressed markers characteristic of cardiomyocytes, such as cardiac α-myosin heavy chain, cardiac troponin I and T, atrial natriuretic factor, and cardiac transcription factors GATA-4, Nkx2.5, and MEF-2. Electrophysiology demonstrated that most cells resembled human fetal ventricular

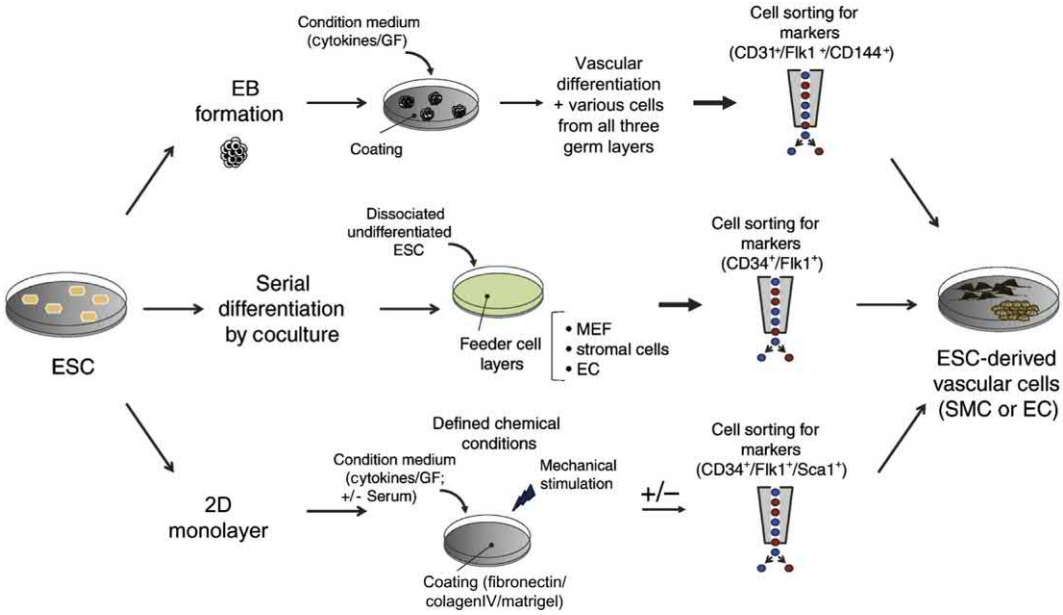

FIGURE 27.3 Approaches used for vascular differentiation of ESCs.
Vascular cell differentiation is mainly induced by three culture methodologies: differentiation through EBs, coculture with fibroblast feeder layers or target cells, and monolayer culture of ESCs in defined chemical conditions. *ESCs*, Embryonic stem cell. *Adapted from Descamps B, Emanueli C. Vascular differentiation from embryonic stem cells: novel technologies and therapeutic promises. Vascul Pharmacol 2012;56(5–6):267–79.*

cells. Despite the progress made over the last decade, knowledge about the mechanism of the formation of functional cardiomyocytes from hESCs remains limited.

Insulin-producing β cells were also generated from hESCs [139], by spontaneous differentiation of hESCs in adherent or suspension culture conditions [140] and using media which contained growth factors [141,142]. Reverse transcription-polymerase chain reaction detected an enhanced expression of pancreatic genes in the different cells [141]. Immunofluorescence and in situ hybridization revealed high percentages of insulin-expressing cells [141].

There has been great interest in examining the osteogenic potential of ESCs derived from both mice and humans. hESCs can differentiate into osteogenic cells (OCs) with the same media supplements that are used to differentiate adult mesenchymal stem cells (MSCs), because of their high self-renewal capability [143]. Current issues associated with the osteogenic differentiation of hESCs include the formation of nonhomogeneous cell populations and limited numbers of differentiated cells. To overcome these limitations, various growth factors and reagents such as β-glycerophosphate, ascorbic acid, dexamethasone, and osteogenic factors have been used to create osteoprogenitor cells from hESCs [144–147]. In addition, the differentiation efficiency of hESCs into a homogeneous OC population was improved through coculturing of hESCs with human primary bone-derived cells in the absence of exogenous factors [148].

The OC-derived from hESCs (OC-hESCs) were seeded on an apatite-coated poly(D,L-lactide-*co*-glycolide)/nano-hydroxyapatite (PLGA/HAp) composite scaffold and subcutaneously implanted in immunodeficient mice to examine in vivo bone formation [149]. The results of in vivo studies demonstrated that the implanted OC-hESCs and apatite-coated PLGA/HAp scaffold induced the formation of large amounts of new bone tissue within the defect site, demonstrating the possibility of using hESCs for bone regeneration [149]. Our group showed that culturing hESCs without EBs leads to an over sevenfold increase in the number of OCs and to spontaneous bone nodule formation after 10–12 days [150]. In contrast, when hESCs were differentiated as EBs for 5 days followed by plating of single cells, bone nodules formed after 4 weeks only in the presence of dexamethasone.

It was shown that the fully decellularized trabecular bones as 3D osteoconductive scaffolds in medium perfused bioreactors cultivated with hESC-derived mesenchymal progenitors led to the formation of large and compact bone constructs. Implantation of engineered bone in immunodeficient mice performed the maintenance and maturation of bone matrix for 8 weeks without any teratomas formation which was largely occurred for groups with undifferentiated hESCs alone or in bone scaffolds. Accordingly, hESC-progenitors can be used in tissue-engineering approaches to grow bone grafts for transitional applications [151].

In another study, native heart ECM was successfully used to direct the cardiac differentiation of hESCs in vitro [152]. Various hydrogels were prepared from decellularized heart ECM blended with collagen type I at varying ratios. Maturation of cardiac function in EBs formed from hESCs was documented in terms of spontaneous contractile behavior and the mRNA and protein expression of cardiac markers. Hydrogel with high ECM content (75% ECM, 25% collagen, no supplemental soluble factors) increased the fraction of cells expressing cardiac marker troponin T (cTnT), when compared to either hydrogel with low ECM content (25% ECM, 75% collagen, no supplemental soluble factors) or collagen hydrogel (100% collagen, with supplemental soluble factors). Native ECM is recognized as a promising biomaterial system since it can induce hESC cardiac differentiation without the need for addition of soluble factors, hence it can be used for basic studies of cardiac development and potentially for the therapeutic delivery of cells to the heart.

Three-dimensional versus two-dimensional cell culture systems

In an appropriate environment, ESCs can differentiate into complex 3D tissue structures. These environments are designed to resemble the key features of the hESC's niche and are favored over the 2D systems, which limit the cellular interactions and signaling and hamper the subsequent differentiation of hESC into functional tissues [6,153]. The use of a biomaterial may act as a temporary ECM, providing physical cues for cell orientation, spreading, differentiation, and the remodeling of tissue structures. Hydrogels represent an attractive option to provide 3D environment. When hESCs were encapsulated in hyaluronic acid, they were shown to retain their pluripotency for 20 days and allow for controlled differentiation [57]. Other types of hydrogels such as chitosan were also shown to preserve pluripotency and self-renewal capacity of the hESCs [154]. The use of alginate hydrogel allowed the preservation of hESC pluripotency for 260 days [58]. It was also demonstrated that the 3D environment created by cell encapsulation in Matrigel failed to support hESC growth and 3D organization, and this was likely due to the fact that Matrigel was unable to resist the force of cell contraction.

It has been demonstrated that the biochemistry, topography, and physical properties of the scaffold can regulate stem cell differentiation and function [6,155]. Culture of hESCs in PLGA scaffolds in specific media containing TGFβ, activin A, or IGF induced the differentiation of the cells into 3D structures with characteristics of developing neural tissues, cartilage, or liver, respectively [156]. Using a chondrogenic medium that contains BMB2 and TGFβ3, it was shown that EBs derived from hESC can lead to the formation of cartilaginous nodules [157]. Cartilage repair was obtained from chondrocytes that were differentiated form ESC-derived MSCs in rats [158]. ESC-derived chondrocytes that were embedded in fibrin gel were also successfully shown to lead to repair of cartilage defects in rats [159].

In the spinal cord the usefulness of ESC-derived neuroprogenitors was demonstrated in one study on experimental dorsal root avulsion injury [160]. Promising results were also obtained from a recent clinical trial employing ESCs for the treatment of spinal cord injury [161]. Oriented Polycaprolactone scaffolds were used to help the differentiation of ESCs to neurogenic lineage [162].

Furthermore, when these cells were cultured in PLGA and poly-L-lactide (PLLA) scaffolds in the presence of media containing nerve growth factor and neurotrophin 3, enhanced numbers of neural structures were observed [163]. In one study, hESC-derived EBs cultured with a 3D collagen scaffold exhibited liver-specific genes expression and albumin production in the presence of exogenous growth factors and hormones [164]. The addition of signaling factors such as activin A and Wnt3a to this system may improve the efficiency of hESCs differentiation and production of functional hepatic endoderm [165].

Similarly, the culture of ESCs in a 3D collagen scaffold, stimulated with exogenous growth factors and hormones, led to the differentiation of the cells into hepatocyte-like cells. These cells were characterized by the expression of liver-specific genes and synthesis of albumin, and the differentiation pattern observed compared favorably to cells differentiated in a 2D system. It was also reported that the differentiation of rhesus monkey ESCs in 3D collagen matrices was different from that which took place in monolayers [166]. Various signaling molecules can be immobilized into matrices and tailor the microenvironment toward desired differentiation [167].

Alginate scaffolds were also used for the differentiation of hESCs [168]. These scaffolds induced vasculogenesis in encapsulated cells to a larger extent than cells grown in bioreactors. Tantalum scaffolds also increased the differentiation of mESCs into hematopoietic cells as compared to traditional 2D cultures [169]. Therefore the 3D culture systems can promote hESC differentiation and assembly into functional tissues, through better mimicking of the 3D structural organization of native tissue compared to 2D systems.

High-throughput assays for directing stem cell differentiation

Today, chemists and engineers are equipped with tools that enable them to synthesize molecules and test their

effects on cells in a high-throughput manner. For example, libraries of small molecules, polymers, and genes have been generated and used to screen candidate molecules to induce osteogenesis [170] and cardiomyogenesis [171] in ESCs as well as the dedifferentiation of committed cells [172]. The use of chemical compound libraries may provide a method of addressing the complexities associated with native microenvironments by directing cell behavior through interacting with transcription factors and cell fate regulators.

Microscale technologies can facilitate high-throughput experimentation and provide a powerful tool for screening whole libraries of molecules and biomaterials. To produce microarrays, where cell–matrix interactions can be tested and optimized in a high-throughput manner, robotic spotters that are capable of dispensing and immobilizing nanoliters of material were used. For example, arrays of synthetic biomaterials have been produced to investigate the interaction of stem cells with various external signals [173]. Using this method, it was possible to synthesize thousands of polymeric materials and investigate their effects on the differentiation of hESCs [174] and human MSCs [175]. Unexpected and novel cell–material interactions were discovered. In future, this technology may prove useful for application in cell-microenvironment studies and in the identification of cues that can be used for the induction of desired cell responses.

In addition to the use of this method for analyzing synthetic material libraries, it is also possible to study the effect of natural ECM molecules on the fate of cells, in a high-throughput manner [173]. In one example, combinatorial matrices comprising different natural ECM proteins were investigated for their ability to maintain differentiated hepatocyte function and to induce murine ESC differentiation into hepatocytes [176]. Recently, Huang et al. used a microscale direct writing technique to print ECM components (e.g., collagen IV, gelatin, and fibronectin) into diverse geometries and compositions on 2D surfaces for assessing the effect of ECM geometry and composition on ectodermal differentiation of murine ESCs [177]. It was shown that ECM compositions, soluble factors, and surface topography could regulate ESC attachment and differentiation [177].

Microfabrication techniques have been also used to control cell–cell interaction and to form hESC-derived EB aggregates with defined sizes and geometries. For example, in one study, soft lithography was used to fabricate cell-repellant poly(ethylene glycol) (PEG) microwells for the formation of EB aggregates with controlled sizes and shapes, determined by the geometry of the microwells (Fig. 27.4A). The EB cell aggregates formed within the microwells remained viable and maintained their geometries over at least 10 days of culture. Using this system, the EB aggregates could pattern into various shapes and sizes (Fig. 27.4B–D) [178]. To control the shape and direct the differentiation of EBs, RA-loaded PLGA microspheres were used to deliver morphogenic factors within EB microenvironments in a spatiotemporally controlled manner [179]. Homogenous differentiation of cystic spheroids with a biepithelial morphology was obtained when EBs were cultured on the fabricated microspheres [179].

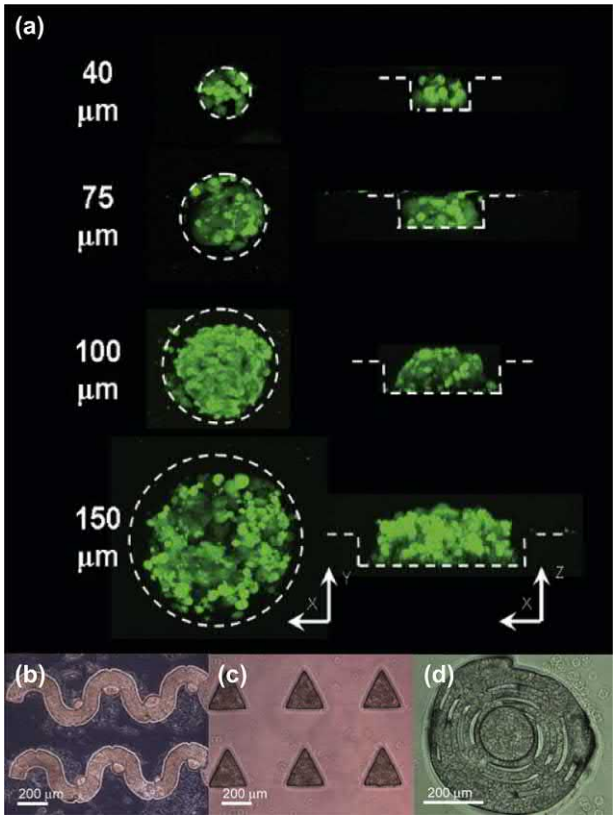

FIGURE 27.4 Microwells for the formation of EBs with controlled size and shape.
(A) Confocal images of fluorescently labeled EB cell aggregates within microwells with different diameter ranging from 40 to 150 μm on day 5 of culture; formation of EBs with different shapes, including (B) curves, (C) triangles, and (D) swirls. *Adapted from Karp JM, Yeh J, Eng G, Fukuda J, Blumling J, Suh KY, et al. Controlling size, shape and homogeneity of embryoid bodies using poly (ethylene glycol) microwells. Lab Chip 2007;7(6):786–94.*

In another study the effect of EB aggregate size on its differentiation was investigated by seeding ESC on PEG microwells of various diameters [180]. It was found that larger microwells (450 μm diameter) induced differentiation of ESC to cardiogenesis through the expression of Wnt11. However, EBs formed in small (150 μm) microwells differentiated to endothelial cell by increased expression of Wnt5a [180].

Cell arrays have been also used to pattern stem cells on substrates. Arrays of cells can be used to localize and track individual cells, which enable clonal analysis of the

fate of stem cells. For example, real-time microscopy was used to track the progeny of neural stem cell clonal populations that were immobilized in microfabricated structures, producing useful information about cellular kinetics and stem cell fate, in a high-throughput manner [181]. It can be, thus, anticipated that such method may be used for investigating the response of individual stem cells to different microenvironmental signals.

Cell patterning on geometrically defined shapes has been used to study the effects of cell shape on cell fate decisions. As cells adhere onto a micropatterned substrate, they align themselves to the shape of the underlying adhesive region. A change in shape induces changes in the cell cytoskeletal features, which in turn influence cell apoptosis, proliferation [182], and differentiation [183]. Coculturing ESCs with secondary cells can promote their differentiation into specific cell lines [184]. For this purpose, Fukuda et al. developed a technique to fabricate micropatterned cocultures of ES with secondary cell lines on surfaces containing three different layers of hyaluronic acid, fibronectin, and collagen [185]. First, the hyaluronic acid was micropatterned on a glass substrate. Then fibronectin was deposited on the areas of exposed glass to create cell adhesive regions. After the cell attachment on fibronectin-coated areas, a layer of collagen was added to hyaluronic acid patterns to switch surface properties and facilitate the adhesion of the second cell type. Using this system, the patterned cocultures of ESCs or AML12 cells with NIH-3T3 could be obtained [185]. Further elucidation of the molecular mechanisms indicated that cell shape regulated the activation of the RhoA pathway demonstrating that stem cell differentiation can be influenced by mechanical stresses. Therefore the use of micropatterning to control cellular microenvironment may be useful for application in directing stem cell fate for tissue engineering.

Physical signals

With advances made in biomaterials, it is becoming possible to influence ESCs by using physical cues such as mechanical forces [186]. Mechanical forces affect the differentiation and functional properties of many cell types and are being increasingly used in tissue engineering. For example, functional autologous arteries have been cultured using pulsatile perfusion bioreactors [187]. Although it is known that mechanical stimuli (such as cyclic stretching and fluid shear stress) may be required to direct the differentiation of ESCs, understanding their effects is still in its infancy [188].

In one study, fluid shear stress was applied to induce Flk-1-positive ES differentiation into vascular endothelial cells through the activation of Flk-1. The expression of vascular endothelial cell-specific markers such as Flk-1, Flt-1, VE cadherin, and PECAM-1 enhanced in the presence of shear stress; however, shear stress had no effect on markers of epithelial or SM (keratin or α-SMA) [188]. In another study, Shimizu et al. demonstrated that cyclic uniaxial stretching on Flk-1-positive ES cells for 24 hours significantly increased the expression of vascular smooth muscle cell (VSMC) markers α-SMA, and SM-myosin heavy chain, decreased the expression of EC marker Flk-1 and had no effect on the other EC markers (Flt-1, VE cadherin, and PECAM-1) [189]. Platelet-derived growth factor (PDGF) receptor beta kinase inhibitor blocked cell proliferation and VSMC marker expression that were induced by applying mechanical stimulation [189]. Mechanical stretching and fluid shear stress have been also used to direct ES cell differentiation into cardiovascular lineages [190,191]. Taken together, these studies demonstrate that mechanical stimulation can enhance the ability of ESCs to respond to exogenous signals and promote their differentiation into a specific lineage. In one study the hESC differentiation on deformable elastic substrates was inhibited by applying a 10% cyclic stretch [65]. The expression of Oct4 and SSEA-4 was promoted in the presence of mechanical stimulation, demonstrating an increase in hESC self-renewal. It was also found that mechanical stretch inhibited hESC differentiation when the cells were cultured in a mouse MEF-conditioned medium. However, differentiation of hESCs was not affected by mechanical stimulation when an unconditioned medium was used [65]. It was also recently found that the mechanical stimulation of ESCs in 3D leads to early expression of cartilage marker gene and to improved differentiation of ESCs to chondrogenic cells [192].

Other environmental factors that may be required include electrical signals. For example, it was found that electrical field stimulation could affect cardiac differentiation and reactive oxygen species generation in hESC-derived EBs [193]. Hopefully, with time, such techniques will allow for the development of ESC-based tissue-engineering applications. The design of bioreactors that control the spatial and temporal signaling that induce ESC differentiation requires further collaborative efforts between engineers and biologists.

Microfluidic systems can be also used to investigate the effect of growth factor and chemical environments on stem cell differentiation in a high-throughput manner. For example, a microfluidic device was developed to generate a concentration gradient of growth factors for optimizing the proliferation and differentiation of stem cells. The developed platform enabled rapid optimization of media compositions by exposing the cells to a continuous gradient of various growth factors within the microfluidic environment to induce proliferation and differentiation in a graded and proportional manner, depending on growth factor concentration [194]. In another study, microbioreactor arrays system comprised a microfluidic

platform, and an array of micro-bioreactors was designed to investigate the effect of culture microenvironments on hESCs differentiation both in 2D and 3D culture conditions [66,195] (Fig. 27.5A–D). Medium perfusion promoted the viability of encapsulated hESCs within hydrogels (67% viability in perfused culture compared to 55% in static culture) (Fig. 27.5E and F). In addition, using this system, it was possible to induce the vascular differentiation of hESCs through the addition of vascular growth factor (hVEGF) to the culture media [195] (Fig. 27.5G and H).

Recently, the role of magnetic particles in guiding stem cell differentiation was reported by Du et al. [97], who used an externally applied magnetic field to pull iron oxide nanoparticle-laden ESCs together and form EBs. These EBs were then exposed to mechanical stretching by using opposing magnetic fields that led the cells to differentiate toward cardiac lineage.

In addition, where both chemical and physical (mechanical) factors are combined to more closely mimic events seen in vivo, new results may be obtained; for example, uniaxial strain was applied to ESC along with chemical stimulus and it was found that myogenic differentiation was significantly higher for group with mechanical loading compared to ESCs alone and ESCs with mechanical and chemical stimuli [196].

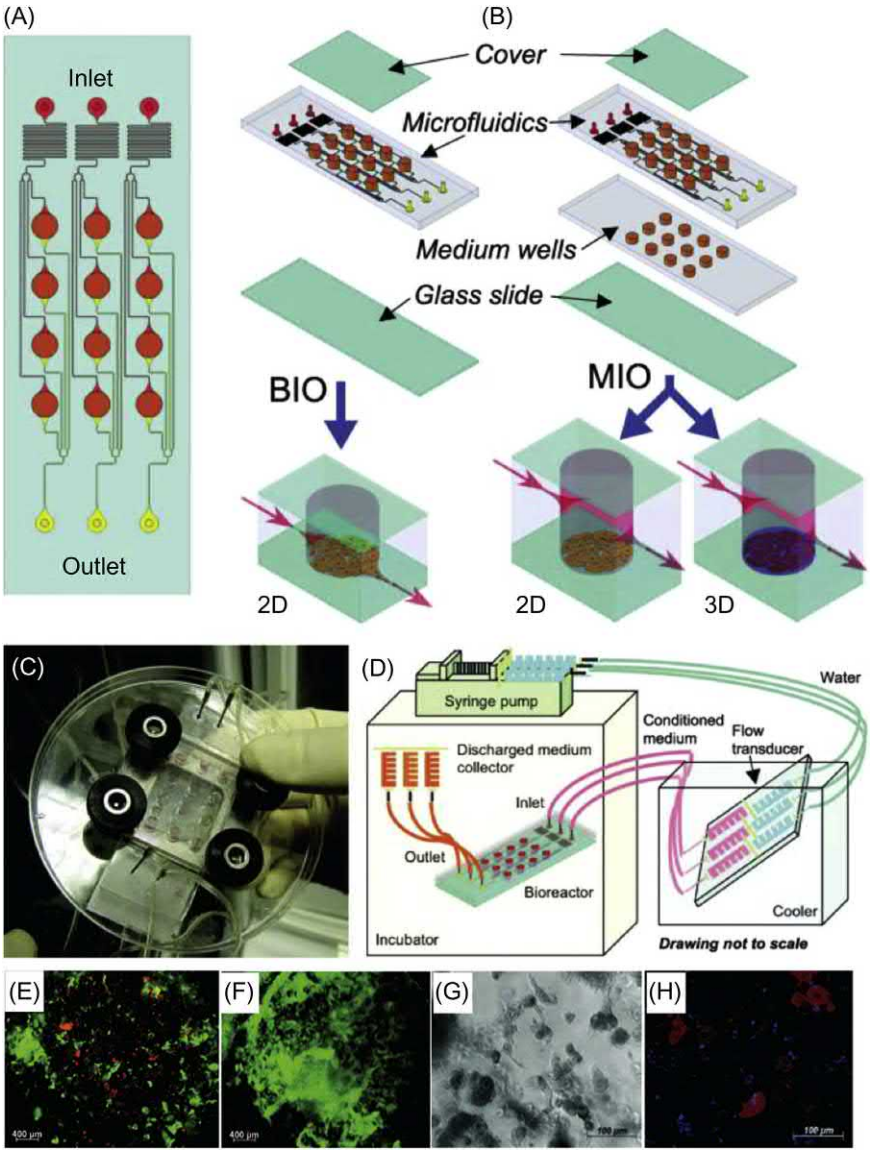

FIGURE 27.5 Microarray bioreactors. (A) The micro-bioreactor wells with 3.5 mm in diameter are arranged in an array. The medium (red) is delivered using three inlets through the flow transducers to four wells (orange) via microfluidic channels (100 mm wide), and waste medium removes from each bioreactor through a separate set of channels (yellow). (B) Two configurations were used: a BIO configuration and a MIO configuration (right) that allows for 3D cultivation. (C) Image of a single MBA with compression frame and fluidic connections. (D) Experimental setup. MBAs and medium collectors are placed in an incubator, and the medium reservoirs are maintained external to the incubator in an ice bath. (E and F) Representative images of hESCs on day 4 of culture (E) without and (F) with perfusion of culture medium (live cells are red and deal cells are green). (G) Bright field image of differentiated hESCs, demonstrating that hVEGF addition to culture media resulted in hESC sprouting and elongation outside the colonies. (H) Confocal image of vascular differentiated hESC indicating the expression of a-SMA (shown in red). *3D*, Three-dimensional; *BIO*, bottom inlet/outlet; *hESCs*, human embryonic stem cell; *MBA*, micro-bioreactor arrays; *MIO*, middle inlet/outlet. *Adapted from Cimetta E, Figallo E, Cannizzaro C, Elvassore N, Vunjak-Novakovic G. Micro-bioreactor arrays for controlling cellular environments: design principles for human embryonic stem cell applications. Methods 2009;47(2):81–9.*

Isolation of specific progenitor cells from embryonic stem cells

Although hESCs can generate specific functional cell types from all three germ layers, it has been exceedingly difficult to directly differentiate the cells in culture and obtain pure cell populations. Isolation of a specific differentiated population of cells for transplantation will eliminate the presence of undifferentiated hESCs, which have tumorigenic potential, and allow for efficient use of the various cell populations for therapeutic purposes. With the exception of few cases, where the enrichment of cells of interest was almost fully achieved [101,106,108], the protocols adopted for the differentiation of hESCs do not yield pure cell populations. Therefore there is a need for suitable techniques to isolate desired cells from heterogeneous cell populations (Table 27.1). One approach for achieving this is to isolate specific cells by using cell surface markers and FACS. In this case, the initial population of cells is immunostained by a single or a combination of different markers, and the desired cell type is isolated by FACS. Part of the initial population of cells is also labeled with isotype controls to gate the populations. The use of FACS yields a pure population of cells and allows one to select cells using different markers [134], but the limitations of this technique may hamper the final cell survival.

Magnetic immunoselection has been used to isolate specific differentiated cells [103,135,204]. Initially, the cells are labeled with relevant cell surface antibodies conjugated with magnetic beads. The magnetically labeled cells are then separated from the other ones by a magnetic

TABLE 27.1 Summary of methodologies to enrich specific lineages from human embryonic stem cells.

Cell type	Methodology followed to enrich specific lineages	Cell lines	References
Cardiomyocytes	Flow-activated cell sorting	hES2	[197]
Cardiomyocytes	Flow-activated cell sorting, magnetic immunoselection	hES2, 3	[198]
Cardiomyocytes	Introduction of a reporter gene and cell selection by flow-activated cell sorting	hES3Nkx2.5^{eGFP}	[199]
Cardiomyocytes	Flow-activated cell sorting	KhES1	[200]
Cardiomyocytes	Flow-activated cell sorting	cmESC, KhESC1, 2, 3	[201]
Cardiomyocytes	Flow-activated cell sorting	hES2	[197]
Cardiomyocytes	Discontinuous Percoll gradient	H1, H7, H9	[138]
Cardiomyocytes	Enzymatic and mechanical dissociation	N/A	[202]
Cardiomyocytes	Enzymatic dissociation	HES2	[137]
Cardiomyocytes	Mechanical (using magnetic field)	CGR8	[97]
Hematopoietic progenitor cells	Magnetic immunoselection	H1, H1.1, H9.2	[135]
Hematopoietic progenitor cells	Flow-activated cell sorting	H1, H9	[203]
Hematopoietic progenitor cells	Magnetic immunoselection	H1, H9	[204]
Leukocytes	Selective adhesion of cells	H1	[205]
Endothelial cells	Flow-activated cell sorting	H9	[132]
Endothelial-like cells	Flow-activated cell sorting	H1, H9	[134]
Neurons and glia	Magnetic immunoselection	H1, H7, H9	[102]
Neurons and glia	Enzymatic dissociation and selective adhesion of cells	H1, H9, H9.2	[103]
Oligodendrocytes	Selective adhesion of cells	H7	[206]
Hepatocyte-like cells	Introduction of a reporter gene and cell selection by flow-activated cell sorting	N/A	[207]

column to purities that are generally higher than 80% [204]. Although these purities are slightly lower than the ones obtained by FACS, the magnetic selection is less harmful to the cells than FACS. Recently, different surface markers specific for cardiomyocytes have been identified: Emilin2 [197] and later SIRPA [198,199] and VCAM [199,200]. These findings allow the prospective isolation of live cardiomyocytes, from ESCs or iPSCs-differentiated mixed cardiac population, with purities above 95%.

Another potential method for cell isolation is through reporter gene knock-in modifications [207]. For example, to trace hepatic-like cells during differentiation of hESCs in culture, a reporter gene expressed under the control of a liver-specific promoter was used [207]. For that purpose, hESCs underwent stable transfection with eGFP fused to the albumin minimal promoter sequence. This methodology allowed one to follow the differentiation pattern of hESCs into hepatic-like cells and to isolate those cells by FACS using the fluorescence of eGFP. Similarly, hESCs are genetically manipulated to carry the Nkx2.5-eGFP reporter construct, allowing the isolation of cardiac cells [199]. Since Nkx2.5 is an early cardiac transcription factor, it allows the identification and isolation of early cardiac progenitors.

Isolation of a specific differentiated population of cells may also be accomplished by mechanical/enzymatic separation of cells exhibiting specific morphology, functional activity, or adhesion to a substrate. For example, cardiomyocytes have been isolated by dissecting contracting areas in EBs and dissociating those areas using collagenase [208]. Oligodendroglial cells were isolated from stem cell aggregates that adhered to a specific substrate [108]. In addition, neuroepithelial cells were isolated from EBs attached to a tissue culture-treated flask by using dispase [103], an enzyme that selectively detached neuroepithelial islands from the EBs, leaving the surrounding cells adhering. It is also possible to take advantage of the cell body content of specific cell types. For example, cardiomyocytes have a higher mitochondrial density than their progenitors and other cardiovascular cells, such as SM and endothelial cells. It is, thus, possible to isolate cardiomyocytes by flow cytometric sorting using the mitochondrial dye tetramethylrhodamine methyl ester percholate without genetic modification or surface antigen staining [201].

Transplantation

The first application of stem cells as a cellular replacement therapy is associated with bone marrow transplantation and blood transfusion in which donor hematopoietic stem cells repopulate the host's blood cells [209]. Today, modalities are being developed for cell-based regenerative therapies of numerous diseases, including diabetes, Alzheimer's disease, Parkinson's disease, multiple sclerosis, spinal cord injury, liver failure, muscular dystrophy, bone, and cardiovascular disease, among others [82]. Despite the advances in the development of disease models [210], only a few studies have reported the in vivo functionality of hESC-derived cells. In most cases the cells are injected into a disease area, and their functionality is evaluated by immunohistochemistry and functional tests. Using such methods, partial functional recovery of a mouse model of Parkinson's disease after hESC-derived neural progenitor cells has been reported [106]. Also, transplantation of hESC-derived oligodendroglial progenitor cells into the *shiverer* model of dysmyelination resulted in myelin formation [211].

Studies of neural regeneration in animal models have given very promising results [212–214]. In particular, hESC-derived oligodendrocytes have been shown to repair injured spinal cord in animal models with rebuilding myelin sheets [215]. Based on this system, in 2010 Geron started the first clinical trial for the treatment of patients with spinal cord injury. A clinical trial also started for the treatment of Stargardt's macular dystrophy, a pathology characterized by the death of photoreceptor cells in the central part of the retina (called the macula). This trial was based on the promising observation that hESCs are able to differentiate into retinal pigmented cells [216–218].

A new important step toward the clinical application of hESCs for infarct therapy is the finding that hESC-derived cardiomyocytes electrically couple and are protective against arrhythmias in the recipient heart when transplanted into guinea pigs [219], an animal model with a much closer heart physiology to humans than that of rodents.

Despite the ability of stem cells to differentiate into cells with desired phenotypic and morphological properties, there have been few scaffold-based tissue-engineering studies that use ESCs, by differentiating these cells in culture, selecting desired cell types, and seeding these into scaffolds. Ideally, scaffolds provide cells with a suitable growth environment, facilitated transport of oxygen and nutrients, mechanical integrity and suitable degradation. The scaffold brings the cells into close proximity and thereby enhances the formation of tissue structures.

Tissue-engineering scaffolds are comprised either synthetic or natural materials or a composite of the two. Scaffolds are commonly made of synthetic materials, such as hydroxyapatite, calcium carbonate, PLLA, polyglycolide, PLGA, poly(propylene fumarate), and natural materials such as collagen, Matrigel, or alginate. Natural materials typically have better biocompatibility, while synthetic materials provide better control of various

properties such as degradation rate, biomechanics, and structure [15]. hESC-derived endothelial progenitors were seeded onto highly porous PLGA biodegradable polymer scaffolds to form blood vessels that appeared to merge with the host vasculature when implanted into immunodeficient mice. These endothelial progenitor cells were also able to support the formation of vascularized skeletal muscle [220]. Osteoblast-like cells derived from hESCs were also transplanted into an animal model by using a poly(D,L-lactide) scaffold. After 35 days, regions of mineralized tissue could be identified within the scaffold by Von Kossa staining and expression of human osteocalcin [144]. For cardiac tissue engineering, synthetic materials were used in the form of injectable hydrogels and surfaces that can be treated to get detached from cardiomyocyte layers [221,222]. Recently, clinical usefulness of ESC-derived cardiac progenitor-laden fibrin was shown [223], including the improvement in their symptoms and systolic motion after ischemic cardiac dysfunction [224]. It was also recently shown that ESC-derived pancreatic progenitors that were encapsulated in alginate were possible to implant and maintain their viability, insulin production, and regulation of glucose levels for over 170 days in mice [225].

Transplantation and immune response

One of the major obstacles for successful transplantation of hESC-derived differentiated cells is their potential immunogeneity. As long-term immunosuppressive therapy would limit clinical applications, the creation of immunologic tolerance would enable stem cell–derived therapy. Methods currently under development include

1. the establishment of hESC line banks large enough to represent the majority of tissue types;
2. nuclear reprogramming of the cells to carry patient-specific nuclear genome (therapeutic cloning);
3. creation of "universal cells" by manipulating the major histocompatibility complex (MHC) [185];
4. deletion of genes for immune response proteins using homologous recombination (as mentioned earlier); and
5. the generation of hematopoietic chimerism, to create the required tolerance for tissues or cells derived from it [226].

The latter method was demonstrated using rat embryonic-like stem cells that permanently engrafted when injected into full MHC mismatched rats [227].

Although the rejection of ESC-derived tissues is triggered by minor histocompatibility antigens, simple host conditioning with monoclonal antibodies against CD4 and CD8 could be sufficient to induce transplantation tolerance of ESC-derived donor tissue, but not of primary animal tissue [228]. It was recently observed that hESCs-derived mesenchymal progenitors have strong immunosuppressive properties [229], similar to bone marrow-derived MSCs [230], resulting in the inhibition of CD4 + or CD8 + lymphocyte proliferation and being more resistant to natural killer cells.

As an alternative to the novel approach of iPSC generation for autologous transplantation or disease-specific drug screening, therapeutic cloning or somatic cell nuclear transfer (SCNT), the process through which Dolly the sheep was cloned in 1997, might be an important tool to create hESCs from patient-specific genome and thus preventing immunorejection [231] (Fig. 27.6).

This is important for the application of hESCs in tissue engineering and regenerative therapeutics [232] where transplantable populations of cells can be generated with genes that are derived only from the patient. Studies to

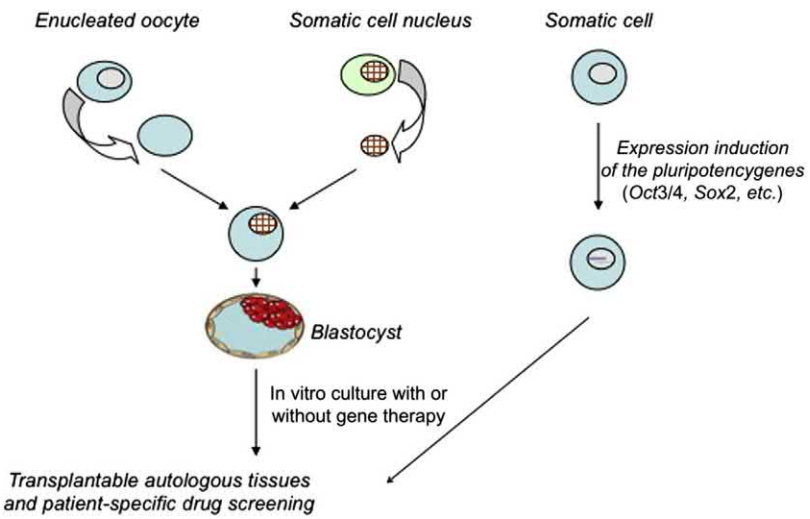

FIGURE 27.6 Schematic diagram of therapeutic cloning.
A somatic cell nucleus is transferred into enucleated oocyte to form a cell capable of giving rise to a blastocyst, which can be used to derive therapeutic cells. Alternatively, somatic cells are reprogrammed into iPS cells that can also be differentiated into therapeutic cells. In both cases, the resulting cells are used to engineer tissues for regenerative medicine and drug screening applications. *iPS*, Induced pluripotent stem.

date have demonstrated that cells derived by SCNT can be expanded in culture and will organize into tissue structures if transplanted with biodegradable scaffolds. However, before SCNT research can be translated into human therapies, the reliability of the overall process needs to be improved, including prevention of the alterations in gene expression.

Immunoisolation systems may help overcome the problems with the immunological incompatibility of the tissue. Thus immunoisolation of cells may prove to be particularly useful in conjunction with ESCs to overcome the immunological barrier associated with the ESC-based therapies. Cells may be immobilized within semipermeable polymeric matrices that provide a barrier to the immunological components of the host. Membranes can be designed to be permeable to nutrients and oxygen while providing a barrier to immune cells, antibodies, and other components of the immune system, by adjusting the cutoff size of membrane pores [233,234]. Within these systems, the engineered tissues can either be implanted or used as extracorporeal devices. Such closed tissue-engineering systems have been used for the treatment of diabetes [235–237], liver failure [238–240], and Parkinson's disease [241–244]. For example, ESC-derived β-cells that can respond to insulin or dopamine-producing neurons can be used in clinics without rejection. In addition, closed systems can protect the host against potentially tumorigenic cells.

Currently, engineering and biological limitations such as material biocompatibility, molecular weight cutoff, and immune reaction to shed antigens by the transplanted cells are some of the challenges that prevent these systems from widespread clinical applications.

Future prospects

Despite significant progress in the field of tissue engineering and ESC biology, there are a number of challenges that provide a barrier to the use of ESCs for tissue engineering. These challenges range from understanding cues that direct stem cell fate to engineering challenges on scale-up, to business questions of feasibility and pricing.

Although the derivation of hESCs from the ICM of preimplantation blastocysts has become a standard procedure and has been performed in a variety of laboratories, live human embryos must be destroyed in the process, which is ethically unacceptable. However, recent reports show that ESCs can be isolated without destroying blastocysts [245]. The generation and use of iPSCs require no embryo at all, overcoming the ethical issues associated with ESCs.

Stem cells and their progeny reside in a dynamic environment during development; thus a scaffold should be designed to mimic the signaling and structural elements in the developing embryo. The use of "smart" scaffolds that release particular factors and/or control the temporal expression of various molecules released from the polymer can help induce differentiation of ESC [246]. For example, by dual delivery of VEGF-165 and PDGF, each with distinct kinetics, and from a single polymer scaffold, resulted in the formation of stable vascular networks [246]. The use of stimuli-responsive materials is another advancing area [247] that can benefit the manipulation and control of stem cell fate and function. An alternative approach to modifying the surface exposed to the cells is to immobilize desired ligands onto the scaffold. For example, arginylglycylaspartic acid peptides, the adherent domain of fibronectin, can be incorporated into polymers to provide anchorage for adherent cells.

Another difficulty with the currently used materials is limited control over the spatial organization of the scaffold. Spatial patterning is necessary to create tissues that resemble the natural structure of biological tissues. In the direct cell patterning system, cells can be seeded into the scaffold at particular regions within the cells. For example, the direct attachment of two different cell types in different regions of the scaffold has been used to generate cells of the bladder. Cell patterning was critical for the effective coculture of hepatocytes and fibroblasts. In addition, the technique of 3D bioprinting has advanced to offer more control over precise localization of cells and active molecules in 3D-printed constructs [248] that can be further used to develop more sophisticated engineered tissue constructs that can be sued for precision medicine. When stimuli-responsive materials are used in 3D printing, a new generation of four-dimensional dynamic engineered constructs can be developed [249]. With the utilization of various molecules, smart materials, and advanced fabrication techniques, it will be possible to leverage the potential stem cells for engineering of tissue constructs.

Stem cells can be used in different 3D bioprinting techniques that include inkjet, extrusion, and laser-assisted printings [250]. Using laser direct writing, EBs with different sizes were formed by using mESCs with controlled initial cell density. The size of the printed colonies was independent of EBs, which represents a viable tool for creating well-defined stem cell microenvironments [251]. Studies have shown that ESCs can maintain their pluripotency following their printing using laser direct writing [252]. 3D bioprinted ESC-laden hydrogel holds promise for use in expansion of ESCs to form pluripotent, high-throughput, and size-controllable EBs [253]. The technique of 3D bioprinting offers new possibilities to expand the potential of stem cells and their use for engineering of tissue constructs that can be used in turn, for the production of tissue models or for regenerative therapeutics.

Conclusion

A number of challenges are still ahead of us before ESC-based therapy can become clinically viable. These include directing the differentiation of ESCs (i.e., using controlled microenvironments or genetic engineering), ensuring their safety (i.e., by eliminating tumorogenicity), functionally integrating differentiated cells into the body, achieving long-term immune compatibility, and improving the cost and feasibility of cell-based therapies. Each of these challenges is currently being addressed. In particular, since ESCs can give rise to many different cell types, solving these challenges for the various possible tissue types will be a major undertaking. Further research is required to control and direct the differentiation of ESCs, in parallel with developing methods to generate tissues of various organs, to realize the ultimate goals of tissue engineering. We might be getting close to a day when ESCs can be manipulated in culture to produce fully differentiated cells that can be used to repair and regenerate specific organs. Clearly, our ability to overcome these difficulties is not confined within any single scientific discipline but rather involves an interdisciplinary approach. Solving these challenges could lead an improved quality of life for a variety of patients that could benefit from tissue-engineering and regenerative therapy approaches.

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Conflicts of interest

During the drafting of this manuscript, JMK has been a paid consultant in the field of regenerative medicine for companies including Stempeutics, Sanofi, Celltex, LifeVaultBio, Takeda, Quthero, and Mesoblast JMK is also an inventor on a patent that was licensed to Mesoblast. JMK holds equity in Frequency Therapeutics, a company that has licensed IP generated by JMK that may benefit financially if the IP is further validated. The interests of JMK were reviewed and are subject to a management plan overseen by his institutions in accordance with its conflict of interest policies.

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Further reading

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Part Seven

Gene therapy

Chapter 28

Gene therapy

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Gene therapy uses the transfer of genetic information to modify a phenotype for therapeutic purposes [1] and has evolved as therapy for diseases that can be cured by the transplantation of genetically modified hematopoietic stem cells [2]. The application of gene transfer to tissue engineering has a myriad of possibilities, including the transient or permanent genetic modification of the engineered tissue to produce proteins for internal, local, or systemic use, helping to protect the engineered tissue, providing stimuli for the engineered tissue to grow and/or differentiate, and to deliver gene editing tools. To provide a background for the application of gene transfer to tissue engineering, this chapter will review the general strategies of the gene therapy, detail the gene transfer vectors used to achieve these goals, and discuss the strategies being used to improve gene transfer by modifying the vectors to provide cell-specific targeting, by regulating the expression of the targeted gene, or by permanently changing gene expression by gene editing. The applications of combining gene therapy with stem-cell therapy will be reviewed. Our overall goal is to provide a state-of-the-art review of the technology of gene therapy, including the challenges to making gene therapy for tissue engineering a reality. For details regarding the applications of gene therapy to specific organs and clinical disorders, several reviews are available [1–11]. An adeno-associated virus (AAV)-based gene therapy treatment for inherited retinal diseases such as retinitis pigmentosa and Leber's congenital amaurosis was approved by the FDA in 2017 [12]. Other cell-based gene therapy approaches that have been included in clinical care are the creation of chimeric antigen receptor (CAR) T cells using lentiviral gene transfer of chimeric anti-CD19 to autologous T cells [13,14].

Strategies of gene therapy

The basic concept of gene therapy is to transfer nucleic acid, usually in the form of DNA (or RNA in retrovirus and lentivirus vectors), to target cells. The vector with its gene cargo can be administered ex vivo, where the gene is transferred to the cells of interest in the laboratory and the genetically modified cells restored to the patient, or in vivo, when the nucleic acid is administered directly to the individual (Fig. 28.1). Independent of the overall strategy, an expression cassette containing the genetic sequences to be delivered, typically a cDNA along with regulatory sequences to control expression, is inserted into a "vector," a nonviral or viral package used to improve efficiency and specificity of the gene transfer. Together, the choice of vector, the design of the expression cassette, and the coding sequences of the gene determine the pharmacokinetics of the resulting gene expression.

Simple in concept, gene transfer is complex in execution. In addition to choosing an ex vivo versus in vivo strategy, the major issues relating to successful gene therapy are the design of vector, how the vector is delivered to the cell population of interest, translocating the vector/expression cassette from outside the cell to the nucleus, and the expression of the gene to obtain the desired therapeutic effect (Fig. 28.2). Independent of the choice and design of the gene transfer vector, successful gene therapy requires decisions regarding the quantity of vector required to modify the numbers of target cells necessary to obtain the desired therapeutic effect and whether or not the vector will evoke a host immune response and/or cause unacceptable toxicity. The gene therapist must also decide how to get the vector to the target cells, including

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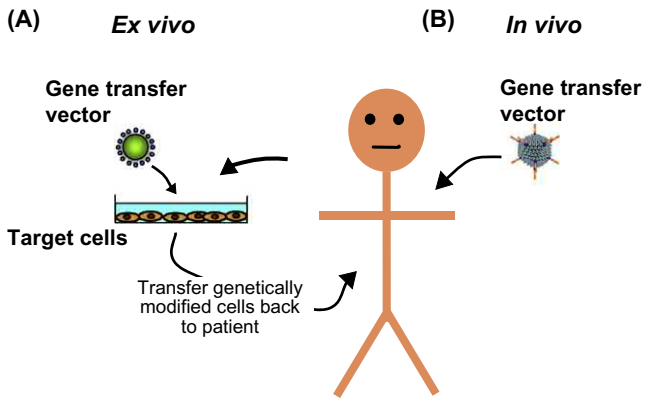

FIGURE 28.1 General strategies for gene therapy for tissue engineering. (A) Ex vivo strategies use a gene transfer vector to genetically modify autologous target cells (e.g., skin fibroblasts) in vitro followed by transfer of the genetically modified cells back to the patient. (B) In vivo strategies transfer the therapeutic gene by direct administration of a gene transfer vector to the patient.

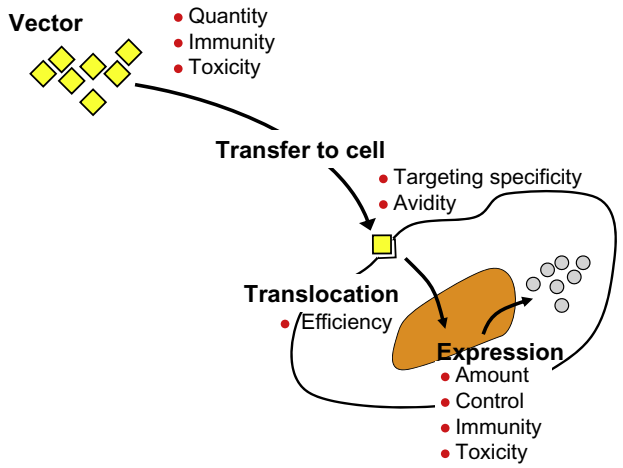

FIGURE 28.2 Issues relating to successful gene transfer. In addition to the choice of vector, successful gene transfer requires decisions regarding the quantity of vector to be used, and that the vector does not induce immunity and/or toxicity that will limit its use. The vector has to be transferred to the cell, a relatively easy task for ex vivo strategies but in vivo may require enhancing targeting specificity and vector avidity to its receptor. Once reaching the target cells, the vector must insure its gene cargo is efficiently translocated to the nucleus. In the nucleus, the gene must be expressed in appropriate amounts, with control if necessary, and without appreciable immunity and/or toxicity induced by the gene product.

targeting specificity and avidity of the vector for its relevant receptor. Once the vector reaches the cells of interest, the gene cargo within the vector must be translocated from outside of the cell to within the nucleus. In designing the gene transfer strategy, it is critical to decide whether or not the gene is to be inserted into the chromosomal DNA of the target cells. Finally, the transferred gene must be expressed, with the concomitant issues of amount and control of expression, and host immunity and toxicity that may be evoked by the expression of the gene. In the sections that follow, we will discuss all of these issues.

Ex vivo versus in vivo gene therapy

There are generally two strategies by which gene therapy technology can be used for genetic engineering, ex vivo transfer of genetic material with subsequent transfer of the modified cells or tissue to the host, and in vivo transfer, with direct administration of the gene therapy vectors to the patient (Fig. 28.1).

Ex vivo

The ex vivo strategy has the advantage that the cell population can be purified and carefully defined, and the transfer of the gene is limited to that cell population and not to other cells or tissues. The challenge for this approach is that, for most applications, once returned to the patient, the genetically modified cells need to have a selective advantage to modulate the therapeutic goal. For applications where long-term expression of the gene is required and the transferred cells subsequently replicate within the patient, the vectors used to transfer the gene must mediate integration of the gene, so that it persists when the cell divides [1,15].

The ex vivo strategy typically is used to genetically modify hematopoietic cells, such as CD34 + cells derived from bone marrow, or skin fibroblasts [5]. Examples of ex vivo gene transfer strategies include the correction of hereditary immunodeficiencies or hemoglobinopathies with retroviral and lentiviral vectors [2], transfer of the factor VIII gene to autologous fibroblasts to treat hemophilia A and transfer of suicide genes to T cells to control graft versus host disease [16–18] or to deliver therapeutic genome editing tools for a variety of therapeutic targets [9]. For tissue engineering applications, the ex vivo strategy is applicable to providing genes to enhance and/or modulate the growth of the engineered tissue, as well as to protect the engineered tissue from host responses or disease processes. The ex vivo strategy is also the most suitable strategy to use gene transfer to genetically modify stem cells.

Although ex vivo gene therapy can be carried out using cells derived from an nonautologous source, potential immune rejection of nonmatched cells generally requires that autologous, or closely matched donor, cells be used for the tissue engineering strategy. However, the immune system can potentially recognize components of the vector and/or transferred gene product. For ex vivo strategies in general, immune recognition of the gene therapy vector is minimal, as the immune system will not be in contact with the total dose of gene therapy vector

used to transfer the gene in vitro, but only the residue of the vector within the cells to be transferred. There is the possibility of immune recognition via MHC presentation of viral antigens inducing antivector immunity, and this may be responsible for shutdown of gene expression over time.

In vivo

In vivo gene transfer strategies administer the gene therapy vector either directly to the target organ or deliver it via the vascular system into vessels feeding that organ. In vivo gene transfer has an advantage over ex vivo strategies in that it avoids the cumbersome (and costly) process of removing cells from the patient, manipulating the cells in vitro, and returning the genetically modified cells to the patient. Challenges that need to be overcome for in vivo gene transfer strategies include the induction of immunity by the gene transfer vector, transport of the gene therapy vector to the targeted cells/organ, efficient binding of the vector to the cell, translocation of the genetic material to the nucleus, and toxicity and immunity induced by expression of virus and/or transgene peptides.

The retina has so far been an ideal target for the in vivo gene therapy approach because (1) it is in a confined, relatively small space; (2) the gene therapy vector can be administered directly; (3) vectors with natural tropism for retinal cells are available; and (4) it is immunoprivileged. The in vivo gene therapy approach has recently achieved a significant success by achieving correction of the genetic disease Leber's congenital amaurosis [19,20].

Chromosomal versus extrachromosomal placement of the transferred gene

One of critical decisions in strategizing gene therapy is whether the transferred gene is to be inserted into the chromosomal DNA of the targeted cells or is designed to function in an extrachromosomal location within the nucleus. There are advantages and disadvantages to both strategies, and the choice of the strategy is determined by the specific application of the gene transfer.

As described next, some gene transfer vectors (e.g., retrovirus, lentivirus) insert their genome, and hence the transferred gene, directly into the chromosomal DNA of the target cells. This has the advantage that it is permanent, and when the genetically modified cells divides, both daughter cells have the newly transferred sequences, as they are now part of the genome of the genetically modified cell population. This is a desirable feature for applications where persistent gene expression is required, such as for the correction of a hereditary disorder. The disadvantage is that, once inserted, the gene cannot be removed, and thus unless controls are designed into the transferred sequences, cannot be shut down. Equally important is the issue of randomness of where the gene is inserted. If the gene is inserted into a relatively "silent" region of the genome, the resulting gene expression will be low, while gene expression from other regions will be high [21]. This genome regional modulation of the level of gene expression will be different for each targeted cell. While there are some more favored regions of gene insertion depending on the vector characteristics, the population of genetically modified cells essentially becomes a mixed population in terms of where the gene has been inserted. Thus expression may be low for some cells, while other cells may be average or high expressors. More troubling, if the gene is inserted into a region influencing cell proliferation, the result may be uncontrolled cell growth, for example, malignancy. This phenomena, referred to as "insertional mutagenesis," has been observed in experimental animal and human applications of gene transfer [21]. Several strategies have evolved out of these observations to improve the safety of integrating gene therapy vectors [22–26].

Some vectors (e.g., nonviral, adenovirus, AAV) transfer the gene into the nucleus, but mostly into an extrachromosomal location, where the gene is transcribed using the same transcriptional machinery as for genomic DNA [1]. The consequences of this strategy is that as long as the cells do not proliferate, and as long as host defenses do not recognize the genetically modified cell as foreign, expression of the transferred gene will persist. However, if the cell divides, the transferred gene will not be replicated, and gene expression in the daughter cells will eventually wane as proliferation continues. The consequences are transient expression of the gene, a "pharmacokinetic" result that is ideal for some applications (e.g., angiogenesis, most cancer therapies) but not desirable for applications requiring persistent expression (e.g., correction of a genetic disease). Independent of the issue of persistence of gene expression, extrachromosomal placement of the gene has the advantage that insertional mutagenesis and variability of gene expression secondary to variability of chromosomal insertion are not of concern.

Gene transfer vectors

There are two general classes of commonly used vectors for gene therapy: nonviral and viral (Fig. 28.3, Table 28.1). Although a variety of vectors have been developed in both classes, the most commonly used nonviral vectors are naked plasmids and plasmids combined with liposomes, and the commonly used viral vectors are adenovirus, AAV, retrovirus, and lentivirus [1,6,7,31–33].

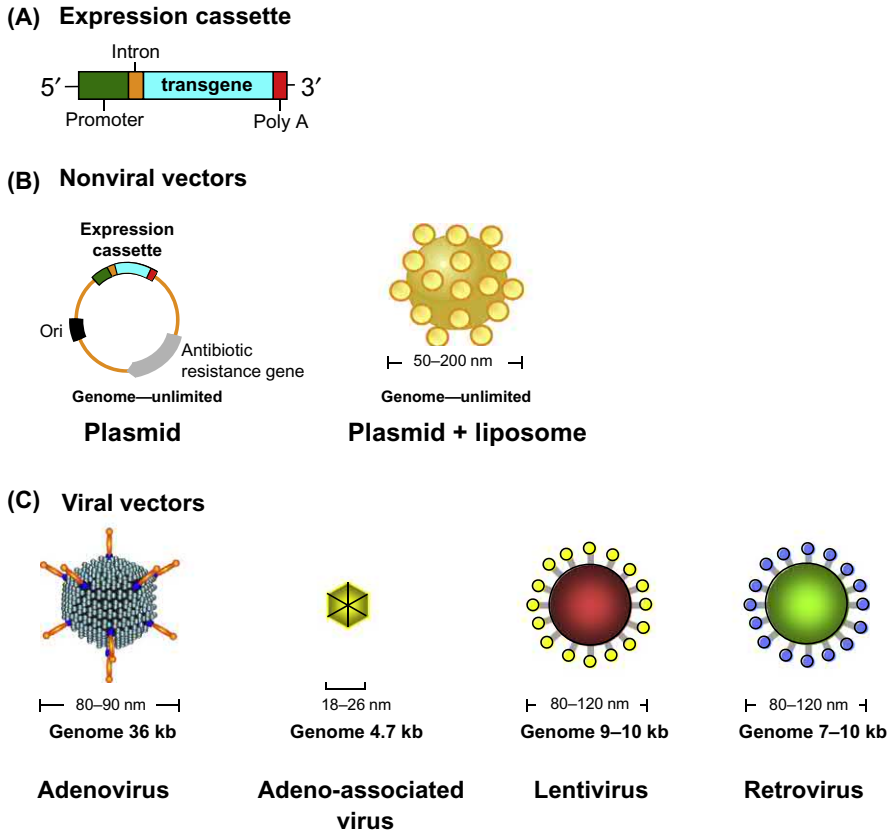

FIGURE 28.3 Commonly used gene therapy vectors. (A) All gene transfer vectors contain an expression cassette with (5′−3′) a promoter, usually an intron, the transgene, and a polyA site/stop signal. (B) Commonly used nonviral vectors, including naked plasmids (typically comprised of an origin of replication, the expression cassette, and antibiotic resistance gene), or plasmid combined with a liposome. The plasmid genome can be unlimited in size, but usually the expression cassette is <10 kb; the liposome/plasmid combination ranges from 50 to 200 nm in diameter. (B) Commonly used viral vectors, including adenovirus, adeno-associate virus, lentivirus, and retrovirus. Shown is the size of the genome of each viral vector, as well as the relative size of each vector. The size of the expression cassette depends on how much of the viral genome is included.

TABLE 28.1 Characteristics of most commonly used gene transfer vectors.

Vector type	Maximum expression cassette capacity (kb)	Transfers genes to nondividing cells	Antivector immunogenicity	Chromosomal integration	Expression	Other characteristics
Plasmids/ liposomes	<10[a]	Yes	None	No	Transient	Poor transduction efficiency
Adenovirus	7−8[b]	Yes	High	No	Transient	Typically, mediates expression for 1−3 weeks
Adeno-associated virus	4.5	Yes	Low	No[c]	Persistent in nondividing cell populations	Expression usually takes 1−3 weeks to be initiated
Retrovirus	8	No	None	Yes	Persistent	Risk for insertional mutagenesis, difficult to produce in high titer
Lentivirus	8	Yes	None	Yes	Persistent	Theoretical safety concerns regarding HIV components; risk for insertional mutagenesis, difficult to produce in high titer

[a]Plasmid size are generally not limited, but plasmids with >10 kb expression cassette capacity are more difficult to produce with consistent fidelity and thus are generally not suitable for gene human transfer.
[b]Typically, Ad vectors have a 7−8 kb capacity for the expression cassette; the capacity can be increased by removing additional viral genes.
[c]Wild-type adeno-associated virus (AAV) integrates in a site-specific fashion into chromosome 19, a process mediated by the rev gene; rev is deleted in the AAV gene transfer vectors; whether or not there is minimal integration of the genome AAV gene transfer vectors is debated [27−30].

Independent of the vector used, all carry an expression cassette, which includes the gene to be transferred together with the relevant regulatory sequences to control the expression of the gene once it has been transferred to the target cells (Fig. 28.3A). The typical expression cassette includes (5′−3′) a promoter, an intron (this is not critical, but it usually enhances gene expression and enables specific polymerase chain reaction (PCR) identification of the cytoplasmic mature mRNA from the pre-mRNA and transferred expression cassette), the transgene itself (usually in the form of a cDNA, but it can contain one or more introns or can be the generic form of the gene), and finally the polyA/stop and other 3′ regulatory sequences, if desired. In special cases, such as *trans*-splicing (discussed in the section on regulation of expression), the expression cassette may contain only a fragment of the gene together with sequences to direct splicing into an endogenous nuclear pre-mRNA.

Nonviral vectors

The most simple gene transfer vectors are plasmids. To achieve relevant transduction of cells in vivo the plasmids are usually combined with liposomes to facilitate attachment and entry into target cells [34,35]. A variety of physical methods have also been developed to promote entry of plasmids into target cells, including microinjection, hydrodynamic administration, electroporation, ultrasound, and ballistic delivery (so-called gene gun) [35]. Most of these physical methods of gene delivery are not applicable for in vivo gene transfer due to the inaccessibility of the target cells to direct manipulation.

Plasmids contain a relatively simple expression cassette with the transgene driven by a promoter and flanked by an intron and the polyadenylation/stop site (Fig. 28.3A). Plasmid DNA has an unlimited size capacity; however, because plasmids >10 kb are potentially unstable, during production most gene transfer strategies being considered for human applications use plasmids <10 kb. Although plasmids can efficiently transduce cells in vitro, their efficiency in vivo is limited. There have been may attempts to correct hereditary disorders with plasmid gene transfer alone but with little evidence of expression of the plasmid-directed mRNA in those trials [36]. Part of the reason of the inefficiency of plasmid-mediated gene transfer is that plasmids have no means to direct their traffic to the nucleus [34]. For gene transfer applications for tissue engineering the most that can be envisioned for the use of plasmid-based systems is to use them in an ex vivo approach with possible need for selection of the transduced cells. There has recently been renewed interest in nonviral gene transfer for the delivery of gene editing systems [37].

Adenovirus

Adenoviruses are nonenveloped viruses containing a linear double-stranded DNA genome of 36 kb [38]. Among the 49 different Ad strains that infect humans, the subgroup C, serotype 5, and serotype 2 are widely used in gene transfer studies and the only serotypes used in humans to date. The Ad5 genome is composed of early and late genes [38]. The E1 region controls the replication of the virus. Conventionally, the Ad gene transfer vectors have a deletion in E1 and E3 (a nonessential region). The expression cassette containing the promoter and the gene to be transferred are usually inserted into the E1 region. The vectors are produced in the 293 embryonic kidney cell line that provides the E1 information in *trans*, enabling replication of the recombinant vector.

Ad vectors can hold 7−7.5 kb of exogenous sequences [38]. If more space is needed, the E2 or E4 region can also be deleted and the vector made in cell lines providing these deleted sequences. The Ad capsid is an icosahedral structure composed of 252 subunits, of which 240 are hexons and 12 are pentons. Hexon is the major structural component of the Ad capsid forming 20 facets of the icosahedron and is composed of three tightly associated molecules of polypeptide II forming a trimer. Polypeptide IX is associated with the hexon protein and serves to stabilize the structure. Each penton contains a base and a noncovalently projecting fiber. Sequences within the fiber are the primary means by which Ad interact with cells, with the penton base providing secondary attachment sequences.

Several cellular receptors have been identified for Ad vectors, and they differ for various serotypes (Table 28.2). The primary Ad receptor for the subgroup C Ad is the coxsackie adenovirus receptor (CAR) [39]. CAR is expressed on most cell types, and thus Ad group C vectors are capable of transferring genes to most organs. Besides the primary CAR receptor, epitopes in the penton base of the group C Ad vectors use $\alpha_v\beta_3$ (or $\alpha_v\beta_5$) surface integrins as coreceptors for virus internalization [40]. Heparan sulfate has also been identified as a receptor for Ad2 and 5, and Ad5 has been shown to bind to vascular cell adhesion molecule 1 (VCAM 1) on endothelial cells and possibly also to MHC class I a2 on the cell surface [44,45].

The group B serotypes Ad11, 14, 16, 21, 35, and 50 utilize CD46 instead of CAR, enabling more efficient gene transfer into hematopoietic cells, cells of the urinary tract epithelium, and salivary glands [47]. For Ad3 of the subgroup B Ad vectors, CD80 and CD86, which are usually expressed on antigen presenting cells, have been identified as a receptor for viral entry [49].

In addition to Ad vectors being effective in delivering genes to a wide variety of cell types for therapeutic

TABLE 28.2 Cell receptors of commonly used viral-based gene therapy vectors.

Vector	Virus group/serotype[a]	Receptor	Reference
Adenovirus	Group C, serotypes 2, 5	CAR; coreceptors—$\alpha_V\beta_3$, $\alpha_V\beta_5$ integrins	[39,40]
	Group C, serotypes 2, 5	Heparan sulfate	[41–43]
	Group B, serotypes 3, 35		
	Group C, serotype 5	Vascular cell adhesion molecule I	[44]
	Group C, serotype 5	MHC class I α2	[45]
	Group D, serotype 37	Sialic acid	[46]
	Group B, serotypes 3, 11, 14, 16, 21, 35, 50	CD46	[47,48]
	Group B, serotype 3	CD 80, CD 86	[49]
Adeno-associated virus	AAV1	Sialic acid (N-linked)	[50,51]
	AAV2	Heparan sulfate proteoglycan; coreceptors—fibroblast growth factor receptor 1, $\alpha_v\beta_5$ integrin	[27–29,52,53]
	AAV3	Heparan sulfate proteoglycan	[41,52,54]
	AAV4	Sialic acid (O-linked)	[54,55]
	AAV5	Sialic acid (N-linked); coreceptor—platelet-derived growth factor receptor	[53,56,57]
Retrovirus	MoMLV	Ecotropic	[58,59]
	470A MLV, VSV	Amphotropic	[58,59]
Lentivirus	HIV-1 (envelope protein)	CD4—coreceptors—CCR5, CXCR4	[60]
	Pseudotyped with VSV-G	Amphotropic (phosphatidylserine)	[61,62]

CAR, Coxsackie adenovirus receptor; MoMLV, moloney murine leukemia virus; VSV, vesicular stomatitis virus.
[a]*Adenoviruses are categorized in groups and serotypes; AAV is categorized in clades and serotypes, only the serotypes are listed.*

purposes, Ad vectors interact rapidly with antigen presenting cells such as dendritic cells, leading to the induction of immunity against the vector and potentially also against the transgene if it is foreign to the host [7,63]. When Ad are directly administered in large doses to animals and humans, there is an innate and acquired immune response against the vector, resulting in local inflammation and infiltration of CD4, CD8, and dendritic cells [63,64]. The immune response is multifaceted consisting of humoral and cellular immunity against both the capsid proteins and against the transgene expressed by the vector if it is foreign to the host. Intravenously administered Ad also interacts with a variety of host proteins, in particular coagulation factor X [65,66].

Several strategies have been investigated to circumvent the problem of host responses evoked against Ad gene transfer vectors, including the use of immunosuppressants administered together with the vector, or including transgenes expressing immunomodulatory factors to suppress the immune responses against the vector [63]. Considerable effort has also been placed on circumventing the host response by designing vectors with larger genomic deletions (e.g., of E1 plus E2 and/or E4 with complementing cell lines) that evoke milder immune reaction [6,63,67].

One challenge to the use of Ad vectors is preexisting immunity against the vector resulting from previous infection with a wild-type Ad virus from the same serotype [63]. The acquired host responses to Ad vector administration generally results in the inability to readminister a vector of the same serotype. To circumvent this issue, alternative serotypes can be administered, thus circumventing immunity against the first vector [68]. Also, different serotypes to which humans are usually not exposed have been developed as gene transfer vectors. One example is Ad serotype 48, to which humans rarely have preexisting immunity. A chimeric Ad vector containing the hexon loops of Ad5 replaced by those of Ad48 has been shown to be a possible strategy for an Ad-based genetic vaccine approach [69,70] and could also be envisioned to be useful for tissue engineering applications.

Another strategy to circumvent preexisting anti-Ad immunity is the use of nonhuman Ad serotypes [71–73]. Nonhuman primate-derived Ad vectors were developed to

overcome preexisting immunity to common human Ad serotypes and to broaden the repertoire of Ad when used as vaccines [74–76]. For example, Ad vectors based on nonhuman primate serotypes C68, C3, C6, C7 do not circulate in the human population and are therefore not affected by preexisting immunity [71–73]. Nonhuman primate-derived Ad strains have been tested as genetic vaccine platforms. The chimpanzee Ad serotype 3 is considered to be one the most efficient platforms for designing new vaccines against Ebola virus [77,78].

Adeno-associated virus

AAV is a single-stranded DNA virus that belongs to the *Dependovirus* genus of the *Parvoviridae* family. AAV were originally isolated as contaminants in laboratory stocks of adenoviruses [79]. Nine subtypes of AAV were initially described for which humans are the primary host [79]. These isolates were found to be different based on the antibody response generated against them and were thus categorized as he AAV serotypes 1–9. A substantial portion of humans have detectable antibodies against these serotypes. To date, more than 100 human and nonhuman primate AAVs have been identified, including 12 serotypes that have between 51% and 99% identity in capsid amino acid sequence [80], but relatively few have been extensively studied for gene transfer [81]. The exact nature and sequelae of the natural infection with AAV in humans are not known, and there is possibly no human disease associated with AAV.

An AAV consists of a single-stranded 4.7 kb genome with characteristic termini of palindromic repeats that fold into a hairpin shape known as inverted terminal repeats [79]. During replication into a double-stranded form, it expresses genes involved in replication [82] and genes that code for the capsid proteins [83]. In the absence of a helper virus such as adenovirus or herpes simplex, wild-type AAV is capable of infecting nondividing cells and integrating its genome into chromosomal DNA at a specific region on chromosome 19, persisting in a latent form. In the gene vectors, *rep* and *cap* are deleted, and most of the vector genome resides and functions in an extrachromosomal locations [79]. In addition to adenovirus and herpes simplex virus, hepatitis B virus [84] and human bocavirus 1 [85], another parvovirus, have recently been identified as new helper viruses. These two new helper viruses may lead not only to a better understanding of AAV–host interactions in the context of chronic infections [31] but may also have implications for novel antiviral strategies or help the development of better AAV production systems [86].

In the AAV vectors the *rep* and *cap* genes are replaced with an expression cassette. During vector production, the *rep* and *cap* gene products as well as the necessary helper-virus elements (usually Ad-derived) are supplied *in trans*. AAV vectors are commonly produced by transfecting two plasmids into the 293 human embryonic kidney packaging cell line. DNA coding for the therapeutic gene is provided by one plasmid, and the AAV *rep* and *cap* functions plus the Ad helper functions are provided by the second plasmid. Titers are generally significantly lower compared with those obtained for Ad vectors but are sufficient to produce enough vector for clinical trials.

Assembly-activating protein, a small and relatively recently discovered AAV capsid protein, plays an important role in the assembly of AAV [87,88] but not for serotypes 4, 5, and 11 [89]. Several small molecules and also larger proteins such as albumin have been shown to enhance AAV infection [90,91]. This may be relevant for improved vector design and better capsid assembly.

Numerous studies in animals have been performed to assess safety and efficacy of AAV-based vectors, and AAV2 serotype-based vectors have been assessed in humans [92]. AAV-vectors are capable of transducing nondividing cells in vitro and in vivo. The exact molecular intracellular state of the vector genome has not been completely elucidated, but there is little evidence that the vectors integrate when used in gene therapy in vivo. Most transgene expression is thought to be derived from extrachromosomal viral genomes that persist as double-stranded circular or linear episomes [93]. This limits the usefulness of AAV for applications involving dividing cells such as stem cells, since only one daughter cell will receive the vector genome [94].

One disadvantage of AAV vectors is their packaging capacity, limited to expression cassettes of about 4.5 kb. This size limitation is a challenge for the use of AAV vectors for clinically relevant large transgenes, such as dystrophin (11 kb) for muscular dystrophy or factor VIII (7–9 kb) for hemophilia A. Various efforts have been undertaken to deliver larger transgenes using AAV vectors, including coadministration of two vectors each carrying one half of the transgene leading to intermolecular recombination during concatamerization, an intermediate state of the vector genome. One approach to overcome transgene size constraint for AAV have been dual-vector approaches, whereby a transgene is split across two separate AAV vectors [95,96].

The host immune response against AAV vectors is not as strong as that observed with Ad vectors [63,97,98]. AAV vectors generate humoral immunity against the capsid proteins, which impairs readministration of vector of the identical serotype. Cellular immunity against AAV has been detected following administration to experimental animals and humans, but, unless high doses are used, there usually are no destructive cytotoxic T-cell responses generated against the vector or the transgene [97]. Innate immune recognition of AAV2 by mouse or human

plasmacytoid dendritic cells is by TLR9 and is independent of the transgene or capsid serotype [99]. The activation of the innate immune response innate immune is through the AAV genome as well as the capsid proteins [98].

Despite these caveats, AAV vectors have so far been the most promising vector system for in vivo administration to humans for the correction of genetic diseases [12,100–105]. Initial trials used this vector system for liver-directed therapy of hemophilia and retina-directed therapy of macular degeneration and other genetic form of blindness. One of the most promising breakthrough has been in patients with spinal muscular atrophy, where a single intravenous infusion of an AAV9 vector containing DNA coding for survival motor neuron 1 resulted in longer survival, superior achievement of motor milestones, and better motor function compared with historical cohorts [105]. Ongoing trials target other severe genetic lysosomal storage disease targeting the brain for mucopolysaccharidosis [106,107], or the heart for Pompe and Danon disease [108].

There is usually persistent expression of the transgene directed by AAV vectors, particularly in organs with nondividing (or slowly dividing) cells, such as liver, muscle, heart, retina, and brain. The overall expression levels of the transgene appear to be lower with AAV compared with Ad and are dependent on the target organ. AAVs that were identified from tissues of nonhuman primates and humans [109,110] have substantial heterogeneity in the capsid genes and are useful as chimeric capsids combine with the AAV2 genome, leading to improved infection of organs or tissues previously not considered to be valuable targets for AAV gene transfer.

AAV interacts with its target cells by binding to cell surface receptors (Table 28.2). For AAV2, the primary attachment receptor is heparan sulfate proteoglycan [27]. AAV3 may share heparan sulfate proteoglycan as the primary attachment receptor. However, AAV3 may use other receptors, as AAV3 has been shown to infect hematopoietic cells, which were not effectively infected by AAV2 [52]. AAV4 and AAV5 use sialic acid as the primary attachment receptor. AAV4 uses O-linked sialic acid, whereas AAV5 uses N-linked sialic acid [55,56]. AAV1 and AAV6 use N-linked sialic acid as receptor [50,51]. Recently, N-linked galactose was identified as the receptor for AAV9 72. The primary receptors for AAV7 and 8 remain unknown. AAV12, originally isolated from a stock of simian adenovirus 18, does not require cell surface heparan sulfate proteoglycans nor sialic acid for transduction and shows strong tropism for nasal epithelia [50,51]. It is being developed as vaccine vector against respiratory pathogens [111].

AAV2 also uses coreceptors for efficient infection, including $\alpha_V\beta_5$ integrin [28] and fibroblast growth factor-1 [29]. Efficient infection with AAV5 appears to require a coreceptor; platelet-derived growth factor has been identified as a possible coreceptor for AAV5 but may also be able to act as the primary receptor [57]. AAVR (AAV receptor; KIAA0319L), a membrane glycoprotein, serves as cellular receptor for several AAV serotypes [54]. This receptor had originally been described in 1996 for AAV2 [112], and its importance and binding capacity for multiple serotypes was recently rediscovered [53]. Further characterization of the AAV binding sites of AAVR identified several polycystic kidney disease domains that can differ for distinct AAV serotypes [113].

Retrovirus

RNA viruses such as retroviruses, lentiviruses, alphaviruses, flaviviruses, rhabdoviruses, measles viruses, Newcastle disease viruses, and picornaviruses have so far been engineered to be used as gene transfer vector for the treatment of a variety of diseases [32]. Retrovirus and lentivirus vectors belong to the family of retroviridae. Because lentiviruses are capable to infect nondividing cells and have evolved as the more promising gene transfer system of the two with transition to clinical care they are discussed separately next. The original retroviral vectors used for gene therapy were based on endogenous murine viruses. Of these the Moloney murine leukemia retrovirus (MMLV) was the first widely used gene transfer vector and was the first to be used to treat a hereditary disorder using an ex vivo strategy [114].

The genome of the retrovirus vector is a 7–10 kb single-stranded RNA containing long terminal repeats (LTR) on both ends that flank *rev*, *gag*, *pol* and other regulatory genes that are required for viral function. The RNA genome of the replication-deficient retroviral vectors contains an expression cassette to up to 8 kb that replaces all viral protein-coding sequences. The LTR's flank the expression cassette and allow transcription initiation by host cell factors. The vectors are rendered self-inactivating by deletion of the promoter and enhancer in the 3′ LTR to prevent LTR-driven transcription. The packaging of the genomic RNA is controlled in *cis* by the packaging signal Ψ. The production of the retrovirus vectors requires a packaging or producer cell line in which the viral *gag*, *pol* and *env* proteins are expressed in *trans* from separate helper products. Recombination between the helper constructs and the vector can be minimized by using nonretroviral regulatory sequences to control expression [115]. Enhancer and promoter sequences can be deleted from the 3′ LTR to create an transcriptionally silent 5′ LTR during infection of the target cells. This strategy provides the basis for self-inactivating vectors and can also be used for the substitution with tissue-specific promoters [116]. The main reason why MMLV

viruses can only infect nondividing cells is that they are unable to cross the nuclear membrane and can only achieve completion of the infection with provirus integration during cell division.

Retroviruses enter the cells via cell fusion of the envelope protein with the cell membrane. The murine viruses are able to infect only murine cells (ecotropic), whereas derivatives of MMLV or human retroviruses like vesicular stomatitis virus (VSV) can infect both human and murine cells (amphotropic; Table 28.2). Providing the virus with a different coat, a process referred to as "pseudotyping," can change the specificity of binding and entry into target cells. Over the past decade, there has been considerable effort in the development of pseudotyping strategies of retroviral vectors [58,61,115,117].

Most of the gene transfer strategies for retroviral vectors have been ex vivo approaches due to the difficulty in producing high titer concentrated preparations and the rapid inactivation of the retroviral vectors in vivo by complement. Due to their ability to infect rapidly dividing cells, retroviral vectors have been used extensively to develop gene transfer strategies to hematopoietic cells. The first clinical trial to treat an hereditary disorder was to use a retrovirus to transduce T cells of patients with adenosine deaminase—severe combined immunodeficiency (SCID) [114], and since then the technology has evolved to permit successful treatment of children with SCID [118–120].

One advantage of using retroviral vectors is the permanent integration of the vector genome into the host genome, providing long-term and stable expression of the transgene. This however also carries the greatest risk of retroviral vectors, in that they may induce insertional mutagenesis with the subsequent development of malignancies. This has been observed in a clinical trial using a MMLV-based retroviral vector to correct hematopoietic stem cells to treat X-linked SCID [16]. Although long-term correction of the immunodeficiency has been observed in the study subjects, three children developed a clonal lymphoproliferative syndrome similar to acute lymphoblastic leukemia. In two of these cases, there was integration of the retroviral vector near the LIM domain only 2 (LMO2) promoter. Retroviruses are currently mostly used for preclinical anticancer strategies. A replicating retroviral vector expressing cytosine deaminase has been effective in preclinical models of glioma [121]. Retrovirus vectors have been useful for gene modifications of natural killer cells [122]. Efforts are ongoing to use retrovirally modified fibroblasts [123] or keratinocyte stem cells [124] for skin diseases such as epidermolysis bullosa.

Lentivirus

Based on the knowledge that the human retrovirus HIV-1 is able to infect nondividing cells such as macrophages and neurons, replication-defective versions of HIV were developed, capable of infecting nondividing cells, and achieve stable long-term expression through integration of the provirus into chromosomal DNA [125]. The genome of a lentivirus vector is a 9–10 kb single-stranded RNA genome, containing components of HIV-1, but otherwise similar to that of retroviral vectors. All viral protein-coding sequences are deleted and are replaced with an expression cassette that can be up to 8 kb in length.

Unique to lentiviruses are the central polypurine tract and central termination sequences, *cis*-acting sequences that coordinate the formation of a central DNA flap which improves nuclear import [126]. This may explain the increased transduction efficiency of lentiviral compared with retroviral vectors. A *rev*-responsive element can be incorporated into the vector to facilitate the nuclear export of unspliced RNA. The packaging of the genomic RNA is controlled in *cis* by the packaging signal Ψ.

Despite the greater complexity the lentivirus genome, the basic principles of generating vectors free of replication-competent virus are similar to those for retroviral vectors. Packaging systems use the HIV *gag* and *pol* genes, with or without the *rev* gene [127,128]. The HIV virulence genes *tat*, *vif*, *vpr*, *vpu*, and *nef* are completely absent from lentiviral vectors, thus making it theoretically impossible that a virus similar to HIV can be inadvertently produced [129]. The production of lentiviral vectors in high concentrated titers is still a challenge, but the production issues are slowly being solved [125]. Lentivirus expression vectors with associated packaging cell lines have been engineered can be efficiently produced in HEK293 suspension cell cultures in bioreactors [130]. Ecotropic [131] and pantropic [132] lentivirus vectors have been designed. The packaging capacity of inserts is similar to what has been described for conventional retroviruses. The chromosomal integration provides long-term expression. Several inducible lentiviral vectors have been designed by the introducing a multiple cloning site upstream of the Tet promoter for the inducible expression of MYC by doxocycline in lung cancer cell lines [133].

The cellular receptors for the lentivirus vectors are the same as for HIV1-1, including CD4, together with the chemokine coreceptors CCR5 and CXCR4 (Table 28.2). However, the lentiviral gene transfer vectors are usually pseudotyped with envelopes from other viruses, such as VSV-G, mediating the binding and entry into target cells similar to retroviral vectors. Efficient gene transfer of lentiviral vectors has been reported for a variety of dividing and nondividing cell types, including muscle, neurons, glia, and hematopoietic cells. Lentivirus vectors have been successfully used to correct disease phenotypes in experimental animals for CNS disorders such as metachromatic leukodystrophy using in vivo administration of the lentiviral vector to the brain [134] or using an ex vivo

strategy by infecting hematopoietic stem cells with the β-globin gene to correct β-thalassemia [135]. There have only been a few clinical studies using lentiviral vectors, but the results for an ex vivo approach correcting hematopoietic stem cells with lentiviral-mediated gene transfer in patients with adrenoleukodystrophy, a severe demyelinating disease, or thalassemia have been very promising [136–138].

Like retroviral vectors, insertional mutagenesis is a concern for the clinical use of lentiviral vectors, and liver cancers have been observed in mice infected in utero or neonatally with lentiviral vectors [139]. One hypothesis for this phenomenon is based on potentially oncogenic sequences present in the woodchuck hepatitis virus–derived posttranscriptional regulatory element that was included into the vector to increase mRNA stability [139]. Lentivirus-transduced autologous hematopoietic stem cells, transduced ex vivo with lentiviral vectors, have been used successfully for the treatment of cerebral adrenoleukodystrophy, Fanconi anemia, HIV-related lymphoma, and sickle cell disease [140]. A variety of preclinical studies using lentivirus-mediated gene expression in CD34 + cells show promise for a variety of monogenic diseases such a pyruvate kinase deficiency [141], thalassemia [140], and various forms of SCID [142]. As outlined next, RNA silencing tools can be efficiently delivered by lentiviral vectors [143].

Cell-specific targeting strategies

Viral gene transfer vectors use the receptors of their wild-type versions to enter into cells (Table 28.2). Modification of gene transfer vectors to target specific cell types or tissues is an attractive means to increase the specificity of the vectors to the target cell and may also enable the vectors to infect cells which are usually not infected by unmodified vectors. In general, targeting modifications can be accomplished by either genetic modification of the vector genome to change the properties of the outer surface of the vector or by chemical modification of the vectors via the addition of ligands (Table 28.3). Based on the targeting strategy used, the range of tropism can be widened or narrowed (Fig. 28.4).

Targeting of Ad vectors

Strategies have been developed to redirect Ad tropism and to enhance Ad tropism for cells difficult to transfer genes to because of lack of Ad receptors. In general, Ad vectors exhibit a broad tropism due to the widespread expression of the primary Ad receptor CAR and the secondary integrin receptors $\alpha_V\beta_3$ and $\alpha_V\beta_5$. While the widespread expression of the Ad receptors enables the efficient infection of a wide range of target cells, it poses the problem of unwanted uptake and gene expression in nontarget tissue when the vectors are administered in vivo. There are some tissues that have low expression of CAR (e.g., endothelial cells, antigen-presenting cells and some tumor cells), which limits the use of Ad vectors for these targets.

Most genetic targeting strategies for Ad vectors have been focused of ablating CAR binding and have introduced new peptides or other ligands to the fiber knob domain, the primary site for the interaction of group C Ad vectors with CAR [180]. Fiber modifications to modify target-cell binding of Ad vectors include the introduction of poly(L) lysine to allow binding to heparan-containing receptors and the integrin-binding motif RGD, which is essential for penton-mediated internalization to allow integrin-mediated binding and uptake [145]. Peptides can also be incorporated into other sites of the Ad capsid to achieve retargeting, such as incorporation of RGD into the hexon [181] and poly(L) lysine into polypeptide IX [146].

Another approach of genetic retargeting of virus vectors is to create chimeras of different serotypes, which are known to use different cellular receptors. Replacing the fiber or fiber knob domain of the Ad5-based vector with that of Ad3 or Ad7 has been shown to achieve CAR-independent infectivity [149–151]. Replacement of the fiber of Ad2 with that of Ad17 has led to improved infectivity of airway epithelial cells [152]. Replacement with the fiber of Ad35 achieved improved infectivity of hematopoietic cells [163,182] and Ad16 enhanced infectivity of cardiovascular tissue [153]. Ad5 vectors pseudotyped with fibers of the subgroup D Ad19 and 37 increased the infectivity of endothelial and smooth muscle cells [147]. Recognition peptides for fiber modification have been identified by phage display [183–185], and other complex genetic modifications of the fiber to retarget the Ad vector have been reported [154,186]. These cell-targeting modifications can also be combined with the use of tissue-specific promoters to achieve selective infection and transcription in the targeted cell type. For example, inserting the RGD motif into the fiber has been combined with the use of the endothelial cell-specific Flt-1 promoter resulted in more specific infection and gene expression in endothelial cells [187]. Finally, a capsid-modified Ad/AAV hybrid vector was able to achieve long-term expression in human hematopoietic cells [163].

Besides genetic modification of gene therapy vectors to modify tropism and target vector uptake to a particular cell type, chemical modifications of Ad vectors has been utilized for targeting. Ad vectors have been complexed to cationic lipids, polycationic polymers, or cholesterol to increase the efficiency of gene transfer in vitro and

TABLE 28.3 Alteration of viral gene transfer vector cell targeting by modifying capsid/envelope structure.

Modification	Viral vector	Examples	Purpose/target	Reference
Genetic alteration of capsid/ envelope proteins	Adenovirus	Delete CAR[a]	CAR-independent infection	[144]
		Add RGD[b] to fiber	$\alpha_V\beta_3$, $\alpha_V\beta_5$ integrins	[145]
		Add polylysine to fiber or protein IX	Broadening infectivity by targeting heparan-containing molecules	[145,146]
		Replace Ad5 fiber with Ad19or Ad37 fiber	Improved infection of smooth muscle and endothelial cells	[147]
		Replace Ad5 fiber with Ad37 fiber	Infection of hematopoietic cells	[148]
		Replace Ad5 fiber knob with Ad3 fiber knob	CAR-independent infection	[149,150]
		Replace Ad5 fiber with Ad7 fiber	CAR-independent infection	[151]
		Replace Ad2 fiber with Ad17 fiber	Improved infection of airway epithelial cells	[152]
		Replace Ad5 fiber with Ad16 fiber	Improved infection of smooth muscle and endothelial cells	[153]
		Replace Ad5 fiber with trimerization motif of phage T4 fibritin	Chimeric fiber phage fibritin molecules targeted to artificial receptors	[154]
		Replace Ad5 fiber knob with CAV2[c] fiber knob	Target to CAR-deficient cells	[155]
		Replace Ad5 fiber with fiber of ovine AdV7	Targeting to kidney and "detargeting" of CAR	[156]
		Add VSV[d]-G epitope to Ad5 fiber knob	Targeting tropism to CAR-deficient cell expressing phosphatidylserine	[157]
		Replace 7 HVR of Ad5 hexon with HVR of Ad48	Circumvention of anti-Ad5 hexon immunity for Ad-based HIV vaccine	[69]
	Adeno-associated virus	Pseudotyping entire capsid with different serotype	Targeting tropism based on capsid serotype	[158]
		Pseudotyping with capsid of multiple serotypes	Generate mosaic AAV to target different serotype tropisms	[159,160]
		Addition of L14[e] to AAV2 capsid	Targeting to L14-binding integrins	[161]
		Incorporation of tumor-targeting peptide to AAV2 capsid	Targeting to CD13	[82]
		Insertion of serpin receptor ligand to AAV2 capsid	Targeting to serpin receptor	[162]
		Ad5 capsid with Ad37 fiber and partial AAV genome	Vector with increased capacity and tropism for hematopoietic cells	[163]
	Retrovirus	Pseudotyping entire envelope with envelope of vesicular stomatitis virus, Gibbon ape leukemia virus, murine leukemia virus	Targeting tropism of new envelope (from ecotropic to amphotropic)	[58,115,117]
		Addition of peptides:	Erythropoietin receptor	[164–166]

(*Continued*)

TABLE 28.3 (Continued)

Modification	Viral vector	Examples	Purpose/target	Reference
		• Erythropoietin • Heregulin • Epidermal growth factor	Heregulin receptor Epidermal growth factor receptor	
	Lentivirus	Pseudotyping entire envelope with envelope of vesicular stomatitis virus G	Improves infectivity to muscle	[167]
		Rabies 6 envelope	Improves infectivity to motor neurons	[168]
		Ebola	Improves infectivity for airway epithelial cells	[169]
		Hantavirus	Improves infectivity to endothelium	[170]
		Fowl plague hemagglutinin (with expression of influenza M2 protein)	Improves infection in airway epithelial cells	[171]
Chimerical modifications of capsid/envelope	Adenovirus	Cationic lipids, cholesterol, polycationic polymers	Modify tropism	[172,173]
		Polyethylene glycol	Decrease immunity	
		Coupling of U7 peptide to Ad5	Targeting of urokinase plasminogen activator receptor on airway cells	[174]
		Addition of single chain antibodies	Target antibody ligand-expressing cells	[175]
	Adeno-associated virus	Chemical conjugation of avidin-linked ligands to AAV2	Targeting to ligand receptors	[176]
		Incorporation of bispecific antibody to AAV2	Targeting to human megakaryocytes	[177]
	Lentivirus	Polyethylene glycol	Prolonging half-life in serum by preventing inactivation	[178]
	Retrovirus	Addition of single chain antibodies	Target antibody ligands	[179]

HVR, Hypervariable regions.
[a]*CAR—Coxsackie adenovirus receptor.*
[b]*RGD—Integrin-binding peptide arginine (R), glycine (G), aspartate (D).*
[c]*CAV2—canine adenovirus 2 [27–30].*
[d]*VSV—vesicular stomatitis virus [27–30].*
[e]*L14—integrin-binding motif.*

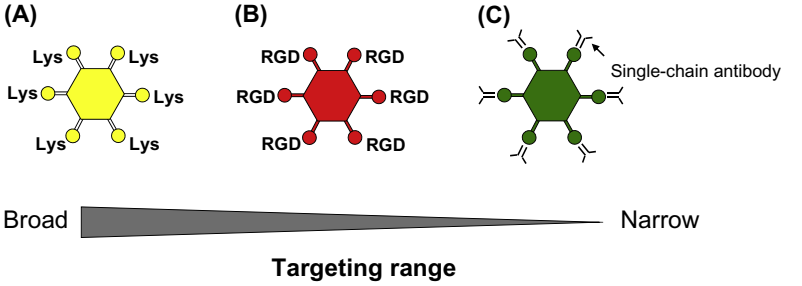

FIGURE 28.4 Examples of modifications of tropism of gene transfer vectors. Shown are three examples of modification of adenovirus vector tropism. (A) The addition of polylysine to the Ad fiber protein provides broad tropism; (B) the addition of the integrin-binding motif RGD enhances targeting of cells expressing $\alpha_v,\beta_{3,5}$ integrins; and (C) the addition of single chain antibodies to the Ad fiber targets cells expressing the antibody ligand. Strategies A→C provide broad to narrow cell-specific targeting.

in vivo [172,173]. Bispecific monoclonal antibodies have been added to the fiber to target specific cell types expressing the ligand for that antibody.

Targeting of adeno-associated virus vectors

The strategy of creating AAV chimeras by pseudotyping with capsids of different AAV serotypes has been used to broaden the tropism of AAV vectors [158–160]. Most of these vectors used the vector genome derived from AAV2 with the capsid from another serotype. For example, this strategy has enabled enhanced transduction in lung-directed gene transfer using an AAV5 pseudotype [188]. The interaction of AAV2 with epidermal growth factor receptor protein tyrosine kinase inhibits transduction and targeted AAV2 for degradation by the proteasome [189]. Mutating tyrosine residues [190] or other kinase targets such as serine, threonine and lysine on the AAV capsid [191] can improve in vitro transduction efficiency and also lead to improved transgene expression [192].

Targeting of retroviral and lentiviral vectors

The classic method to broaden the tropism of retroviral and lentiviral vectors is by pseudotyping, creating chimeras using envelope glycoproteins from other viruses. Most retrovirus vectors are based on MuMoLV, an ecotropic virus, which infects only murine cells. To achieve infection of human cells, the vectors are propagated in packaging cells that express the envelope of the amphotropic or nonmurine viruses such as 4070A murine leukemia virus, gibbon ape leukemia virus, VSV, or the feline endogenous virus RD114 [58,117]. Various envelopes have also been used for lentiviral vectors to increase infectivity of specific cell types, including VSV-G for muscle [167], Ebola for airway epithelial cells [169,193], rabies-G for motor neurons [168] and hantavirus for endothelium [170]. Hybrid proteins of the murine amphotropic envelope have been combined with the extracellular domains of GALV or RD114 envelope to enhance infection of CD34 + cells [194]. As with Ad vectors, strategies have been developed to target retro- and lentiviral vectors by addition of ligands to the envelope glycoprotein. Examples include peptide sequences from erythropoietin [164], heregulin [165], epithelial growth factor and ligands for the Ram-1 phosphate transporter [115] as well as the addition of single chain antibodies [166,179]. The efficiency of these modifications however has not been very high, and the vector production yield is significantly impaired.

Another strategy has been to utilize the membrane proteins that are incorporated during the budding process for targeting. For example, incorporation of the membrane-bound stem-cell factor provided not only a growth signal for the CD34 + cells expressing c-kit, the receptor for stem-cell factor but also lead to increased infection efficiency of the CD34 cells [195]. Monomethoxy poly(ethylene) glycol conjugated to VSV-G protects the vector from inactivation in the serum, leading to a prolonged half-life and increased transduction of bone marrow following intravenous administration in mice [178]. Another strategy for targeting of retro- and lentiviruses in vivo has been to target retrovirus producer cells [196].

Regulated expression of the transferred gene

A variety of strategies have been developed to regulate expression of the genes transferred by gene therapy vectors (Table 28.4). The ability to regulate gene expression is particularly important for application where too much expression of the gene transfer product could lead to unwanted effects, for example, sustained expression of a growth factor that could potentially be tumorigenic. For in vivo strategies of gene transfer tissue-specific regulation of gene expression may be warranted to avoid expression of genes in undesired cells or tissues. In the context of the use of gene transfer vectors to genetically modify stem cells, regulation of gene expression may be critical to avoid differentiation into an unwanted tissue or cell type. The regulation of the gene expression of the transgene could also be combined with inducible systems of suicide genes or factors that could destroy the genetically modified cells, should unwanted differentiation occur.

To turn gene expression on and off at will, a number of inducible promoters and inducible regulated systems have been developed that are applicable to be used in gene transfer vectors (Table 28.4). For example, inducible gene expression in gene transfer vectors can be achieved using inducible promoters such as promoters responsive to glucocorticoids, cGMP, heat shock protein, radiation, and insulin. Inducible regulated systems also include systems based on response to tetracycline, antibiotic resistance, chemical-induced dimerization, steroid receptors, and insect ecdysone receptors. The basic mechanism for these systems is a combination of ligand-binding synthetic inducer or repressor proteins and promoter control system that regulates transgene expression.

The tetracycline-responsive system has been widely used to study gene function and to generate conditional mutants in cell lines and transgenic animals [239]. The transgene is placed behind a promoter that also contains binding sites for the tetracycline response element (TRE), which can act as a repressor or inducer of transgene expression. A fusion protein that binds to the inducer

TABLE 28.4 Regulation of expression of the transferred gene.

Category	Strategy	Note	Reference
Inducible promoter	Glucocorticoid–responsive	Multiple response elements	[197]
	cGMP[a]-responsive	Multiple response elements	[198,199]
	Heat shock protein-inducible	Hyperthermia and cellular stress induce gene expression	[200,201]
	Radiation-inducible		[202]
	Insulin-responsive		[203]
	Tetracycline-responsive	Repressible-TET_{off} or inducible-TET_{on} systems	[204–210]
	Antibiotic resistance	Streptogramin class antibiotic (e.g., pristinamycin) induces pristinamycin-induced protein preventing expression	[211]
		Erythromycin binds to prokaryotic DNA-binding protein MphR(A)[b]	[212]
	Chemical-induced dimerization (FKBP/FRAP[c])	Rapamycin induces heterodimerization of FKBP and FRAP	[213–215]
	Steroid receptor	Transactivator (GLVP[d] or Glp65) targeting genes with GAL-4-binding site in the presence of mifepristone (RU486)	[216,217]
	Insect ecdysone receptor	Ectodysone receptor-ligand induces transactivation of transgene	[218,219]
Tissue-specific promoter	Liver	Albumin, α1-antitrypsin, LAP, transthyretin promoters	[220–222]
	Smooth muscle	Smooth muscle actin, SM-22, smooth muscle myosin heavy chain promoter	[223,224]
	Prostate	Prostate-specific antigen promoter	[225,226]
	Neuron	Synapsin I, neuron-specific enolase	[227]
	Dendritic cells	Dectin 2, vFLIP	[228,229]
	Vasculature	VE-cadherin, smoothelin B	[83]
	Tumor vasculature	Tie2	[230]
Trans-splicing	Therapeutic *trans*-splicing	Target pre-mRNA is *trans*-spliced into independent pre-mRNA	[231,232]
State of differentiation	Differentiation-specific endogenous transcriptional regulatory elements	Beta-globin gene expression by endogenous transcriptional regulatory elements or from other genes with a similar expression pattern	[233,234]
Gene disruption	Nonhomologous end-joining gene disruption by zinc finger nucleases	Disruption of CCR5 gene to create resistance to HIV in T cells and hematopoietic stem cells	[235,236]
MicroRNA	Target cell-specific microRNAs	No transgene expression by incorporating a target sequence for microRNA in the 3′UTR	[237,238]

LAP, Liver-activated protein.
[a]*cGMP—cyclic guanosine monophosphate.*
[b]*MphR/A—a prokaryotic DNA-binding protein that binds to a 35 bp operon sequence.*
[c]*FKBP [30]—FK506-binding protein; FRAP—FK506-binding protein rapamycin-binding.*
[d]*GLVP—a mifepristone-activated chimeric nuclear receptor.*

doxycycline needs to be present on a separate gene construct. The tetracycline transactivator binds to the TRE and activates the transcription in the absence of doxycycline. Upon addition of doxycycline, the expression is turned off (Tet-off). Another fusion protein that can be used is the reverse tetracycline transactivator, which only binds to TRE in the presence of doxycycline, causing induction of expression upon addition of doxycycline (Tet-on). Expression can be controlled in a graded manner; the more doxycycline is added the greater the level of suppression or induction. The disadvantages of this system are the potential side effects of doxycycline. The tetracycline system has been used for regulated gene transfer with gene therapy vectors, including Ad [204,240], AAV [205–207], retroviral [208,241], and lentiviral vectors [209,210].

The steroid receptor systems use a mifepristone-binding progesterone receptor fused to the DNA-binding domain of the yeast GAL4 protein and the transactivation domain from the NfkB p65 subunit [216,217]. The fusion protein binds to a GAL4 activating sequence to regulate gene expression. Upon addition to mifepristone, gene expression is induced and upon removal of the drug gene expression returns to baseline within 5 days.

The insect ecdysone receptor system consists of a fusion protein of the transactivation domain of the glucocorticoid receptor fused the ecdysone-binding nuclear receptor and an ecdysone-response element placed upstream of the promoter driving the transgene expression [218,219]. Upon addition of ecdysone, an insect hormone with no mammalian homologs, the fusion protein dimerizes and induces expression. Because there are no known mammalian factors binding to the insect protein, there is very low background expression in the absence of the drug and expression can be very tightly controlled.

Another strategy to control gene expression at the desired location is the use of tissue-specific promoters (Table 28.4). The majority of tissue-specific promoters have been used to target expression to liver and the cardiovascular system by targeting muscle cells. The challenge in the use of tissue-specific promoters is that the level of expression is usually lower compared with the commonly used strong viral promoters. However, viral promoters such as CMV have been shown to be subject to silencing after several weeks in vivo. This has been seen in airways, cardiomyocytes, and smooth muscle cells in particular with nonviral gene transfer [223].

Another modality to regulate gene expression is by *trans*-splicing at the pre-mRNA level. In therapeutic trans-splicing, the sequence of the target pre-mRNA is modified by being *trans*-spliced to an independent pre-mRNA, the sequences for which are delivered exogenously by a gene transfer vector [242,243]. Therapeutic *trans*-splicing can be used to alter coding domains, to create novel fusion proteins, to direct gene products to various cellular compartments, and to enable gene therapy with large genes or genes coding for toxic products. *Trans*-splicing gene transfer strategies also offer the advantage that the expression of the *trans*-spliced sequence is controlled by endogenous regulation of the target pre-mRNA. *Trans*-splicing strategies have been used to correct animal models of hemophilia, X-linked immunodeficiency with hyper IgM and cystic fibrosis [231,244,245].

Using gene transfer vectors for gene editing

The recent technological advances in gene editing have further broadened the use of gene therapy vectors. Gene editing is applied for the rapid generation of knock-out cell lines or animal models [246], functional genomic screens [247], and other applications of transcriptional modulation/gene silencing [248]. Several viral [249] and nonviral [37] gene delivery systems are currently being explored for in vitro and in vivo delivery of gene editing systems, most commonly clustered regularly interspaced short palindromic repeats (CRISPR), together with CRISPR-associated proteins (Cas). CRISPR/Cas9 are part of the prokaryotic adaptive immune system and have successfully been repurposed for genome editing in mammalian cells [250]. The system generally consists of Cas, a specific endonuclease, and a guide RNA molecule which guides Cas to a specific DNA target [251]. Following translocation across the nuclear membrane, the heterologous complex cleaves a target sequence in the chromosomal DNA. The endonuclease and the guide RNA have to be heterologously expressed, which poses a challenge for their efficient delivery. The class 2 endonuclease Cas9 is the preferred system as it consists of a single protein [252]. Off-target effects of the CRISPR-Cas9 systems in its current form pose a challenge for their safe use in humans. Multiple guide RNAs can be expressed from the same construct to target multiple genes or to enhance the knock-out by targeting multiple sites in the same gene [253].

AAV8 has been used successfully for liver-directed delivery of CRISPR/Cas9 [254]. AAV9 is a suitable vector for delivery of CIRPR/Cas9 to skeletal muscle, heart, and brain [255–257]. However, the low packaging capacity of AAV is not sufficient for the packaging Cas9 and a guide RNA together, which requires around 4.2 kb [258]. This can be solved by using separate vectors for Cas9 gene and the guide RNA with the challenge to deliver both vectors in the same target cell in vivo [259]. A split Cas9 was developed that could be divided over two AAV cassettes and naturally joined after each part of the protein was expressed in the cell with the goal to provide additional space for regulatory elements and/or multiple guide RNAs [260]. A smaller but equally active ortholog of Cas9 from *Staphylococcus aureus* delivered by AAV8 showed efficacy for liver-directed gene silencing in vivo [258].

Lentiviral vectors have also been used to deliver gene editing systems due to their potential to alter cellular tropism by pseudotyping [261] and their ability to integrate into the host genome, which may enhance the risk for off-target effects such as mutagenesis. When nonintegrating, lentiviral vectors are used [10]. Lentiviral vectors are most commonly used to create disease models. Several guide RNA libraries in human and murine cells have been developed by large collaborative genome-wide loss-of-function screening using lentiviral delivery [259,262,263], for mouse embryonic stem cells [264], the identification of targets for West Nile virus [265], gene editing in plants [266], and for the creation of a murine leukemia model [267].

Ad-mediated delivery of CRISPR/Cas9 has been studies to a lesser degree but has nevertheless shown some promise [249]. Ad vectors have been used in animal models to decrease cholesterol levels by creating mutations in mouse liver [268] and in muscle-directed strategies for muscular dystrophy [269,270]. Ad vectors have also been useful in the creation of disease models [271,272]. Ad-mediated delivery of CRISPR/Cas9 to inactivate the SMAD3 gene in human lung fibroblasts and bronchial epithelial cells was studied to create a tool for drug discovery [273].

Nonviral delivery strategies include the use of cell-penetrating peptides [274], a less likely option for in vivo delivery, because the individual components need to be delivered to the same cell simultaneously. DNA nanoclews, nanoparticles based on a cage of DNA that is made by rolling circle amplification, and the complexes Cas9 and the guide RNA [275] are another nonviral vector approach.

To tackle both the caveat for off-targeting and the limited space, strategies have been designed to combine viral and nonviral delivery. A nonviral second vector was used for the delivery of Cas9 mRNA with an AAV8 vector expressing the guide RNA [9].

Combining gene transfer with stem-cell strategies

In general, stem cells are categorized into embryonal stem cells, the bone marrow-derived stem/progenitor cells including mesenchymal stem cells (MSC), endothelial progenitor cells [272], and the tissue-derived stem-cell populations [276]. Stem cells offer the potential for tissue regeneration, and combining gene therapy and stem-cell approaches is a promising strategy to direct the differentiation of stem cells in the desired cell type and to regulate and control growth and differentiation of the stem cell. Stem cells have the potential for both self-renewal and differentiation and are dependent on signals form their microenvironment that direct stem-cell maintenance or differentiation. The precise spatial and temporal presentation of these signals is critically important in development and will most likely be the decisive factor in the success of stem-cell therapies [277]. As the potential of stem cells to be used clinically becomes more realistic, gene transfer strategies may play a pivotal role in controlling stem-cell growth, by either preventing uncontrolled growth and/or directing differentiation into specific cell types and regulating gene expression [278]. In addition, as more of the microenvironmental cues that control stem-cell differentiation into the desired phenotypes become known, the potential to use gene transfer strategies to express or inhibit these signals become a viable strategy to support stem-cell therapy. Three general areas can be currently envisioned for gene transfer to be helpful for stem-cell therapies: (1) to control unwanted stem-cell growth or differentiation; (2) to provide environmental cues for stem-cell differentiation; and (3) to regulate gene expression. In addition gene transfer may also be useful in marking stem cells as a modality to track the cells and their progeny in vivo [279].

Gene transfer to stem cells

The challenges in developing gene transfer strategies for stem cells are (1) how to most efficiently infect the cells, (2) gene silencing during differentiation, (3) if gene expression should be transient or persistent, and (4) if gene expression is persistent should it terminate once the cell has differentiated into the desired phenotype? Gene transfer has been accomplished to a variety of stem-cell types. Human and murine embryonal stem cells have been successfully infected with subsequent gene expression with adenovirus, AAV, and lentiviral vectors [280]. The reported efficiency for each of these vector systems is limited, however, requiring selection to obtain pure populations of genetically modified cells. Gene expression can be affected by subsequent differentiation of the cells, and it is not clear how the stem-cell properties are affected by the various gene therapy modalities themselves without transgene expression. Bone marrow−derived endothelial progenitor and MSC have been successfully transduced with the most commonly used gene therapy vectors [5].

Gene transfer to control uncontrolled stem-cell growth

Gene transfer strategies developed to control the growth of cells for cancer gene therapy applications can be applied to controlling unwanted growth of stem cells, in particular the risk of the development of teratomas with embryonic stem cells. Some of the suicide gene transfer strategies, including the prodrug strategies using herpes-simplex thymidine kinase (ganciclovir) and cytosine-deaminase (5-fluorocytosine) that lead to activation of cell death following administration of the prodrug may prove useful. Other suicide genes may be used with regulatable gene expression systems (as outlined above, Table 28.4) may be useful to control potential malignant stem-cell growth, as long as there is no “leak” of baseline gene expression of the suicide gene.

Gene transfer to instruct stem-cell differentiation

Gene transfer strategies may prove useful in providing the necessary environmental factors necessary to develop

specific phenotypes from stem cells. For example, expression of growth factors or other known differentiation signals secreted by the stem cell should aid in mediating differentiation, that is, the genetically modified stem cell would create its own favorable microenvironment for differentiation. Expression of these factors could be regulated by inducible gene expression systems. For example, for muscle-derived MSC, with their potential for the treatment of skeletal, cardiac and smooth muscle injuries and disease, the cytokines required for the differentiation in the respective lineage have been identified [281].

Gene transfer to regulate gene expression

Regulation of gene expression is critically important in the maintenance and differentiation of stem cells, and the transfer of genes coding for factors that regulate endogenous gene expression in response to specific stimuli could prove very helpful for stem-cell therapies. This concept has been used in gene transfer strategy to regulate angiogenesis in the ischemic myocardium. AAV vector–mediated transfer of a hypoxia a responsive element to ischemic myocardium resulted in endogenous expression of the vascular endothelial growth factor (VEGF) [282]. Similarly engineered transcription factors capable of activating endogenous VEGF expression have been successfully transferred with adenoviral vectors [283]. As more of the gene expression and regulation patterns critical for stem-cell differentiation and maintenance are known, the possibilities to direct or aid in gene expression using gene transfer are significant.

One potential barrier to using embryonic stem cells in humans is rejection of the transplanted cell by the immune system [284]. This could theoretically be circumvented by using gene transfer with the relevant gene to autologous stem cells. For example, transfer of a genetically corrected nucleus to an enucleated egg from an unrelated donor would result in the generation of genetically modified embryonic stem cells that could be then differentiate and correct differentiated cells transplanted to the same patient (Fig. 28.5).

Challenges to gene therapy for tissue engineering

Although proven to be very effective in a variety of model systems, the major challenges for gene therapy to cure human diseases include circumvention of immune responses against viral vectors, transferring the genes to a sufficient number of cells to change the phenotype and controlling the expression of the gene. The main hurdle for successful gene therapy to compensate for a missing or defective protein has been the host response to the gene therapy vector, the lack of long-term gene expression, and problems related to integration into the host genome. However, short-term expression of transgenes has been feasible in humans and shown efficient in a variety of cell types and tissues. Immunogenicity against a nonself transgene, as well as vector-derived proteins, may be an issue if gene transfer is being used for permanent expression of a transgene. Controlling the gene expression is a challenge that needs to be addressed, especially if gene transfer strategies are combined with stem-cell

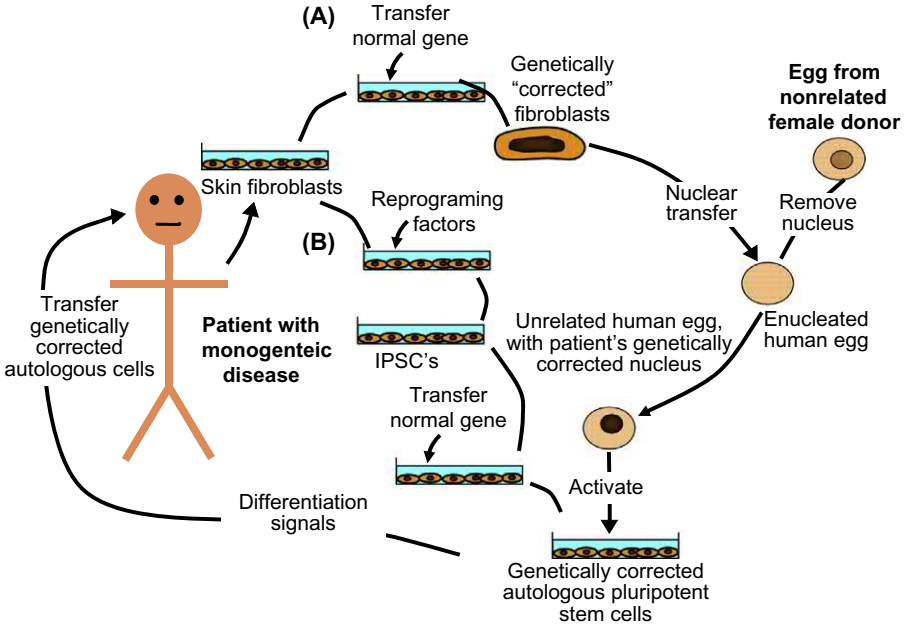

FIGURE 28.5 Strategy to combine gene therapy with nuclear transfer and stem-cell therapy. Shown is an example to genetically modify skin fibroblast of an individual with a monogenetic disease to correct the abnormality. The nucleus of the genetically corrected fibroblast is then transferred to an enucleated egg of an unrelated donor to generate corrected autologous pluripotent stem cells that can be differentiated and then transferred back to patient.

strategies. Most regulatable gene expression systems show some baseline expression, which may be problematic if gene transfer is used to regulate gene expression for stem-cell differentiation.

Over the past decade, progress has been made in addressing many of these challenges that led to clinical approval of several gene therapy vectors for cancer and genetic diseases. Based on the continued focus on solving these issues, together with gene therapy, the knowledge gained from successes and setbacks will also prove beneficial in their use for tissue engineering and stem-cell applications.

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Chapter 29

Gene delivery into cells and tissues

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Introduction

This chapter provides an overview of intracellular gene and nucleic acid delivery by engineered nanoparticles (NPs) and viral vectors, including particle and vector design, strategies for overcoming cellular barriers, and methods for delivery to cells that are particularly relevant to tissue engineering and regenerative medicine. NPs include a variety of engineered, chemically defined, colloidal objects capable of entering cells and tissues and delivering cargo intracellularly. Viral vectors broadly include a variety of infectious particles with diverse properties that can be adapted to tissue engineering and other applications. This chapter focuses on both nonviral and viral approaches for safe and efficacious intracellular delivery of nucleic acids and delivery routes including systemic delivery and local administration to pathological sites.

Fundamentals of gene delivery

The engineering of nucleic acid delivery systems is a valuable approach for tissue engineering and regenerative medicine. Nucleic acid-based drugs are broadly sought to (1) replace missing, mutated, or deficient genes, (2) for RNA interference (RNAi) to silence aberrant, regulatory, or pathologic genes, (3) to control RNA splicing to remove pathogenic exons, (4) to control microRNA (miRNA) activity, or (5) for genome editing to correct endogenous mutations, or to change cell function or cell fate (Fig. 29.1).

Gene replacement or addition is a more mature but still challenging field that involves transfer of DNA that encodes the desired gene typically through viral delivery [1]. Several gene therapy approaches are now approved clinical products with many more in the pipeline. Gene therapy is based on the premise that loss-of-function genetic disease can be reversed by replacing the defective genes. Through nearly 50 years of development, gene therapy is poised to make a substantial impact in medicine in the coming years.

Because of the potency, specificity, and transient activity of RNAi, engineering vectors for delivery of this class of gene therapy has become a major focus of the tissue engineering and medical communities, and significant progress has been made toward technologies that enable therapeutic gene silencing in vivo, including the first clinically approved RNAi therapeutic [2]. Gene expression knockdown can be achieved through several different strategies, the earliest of which was utilization of antisense oligodeoxynucleotides (ODNs), which are single-stranded DNAs that bind to complementary mRNAs and yield a relatively modest reduction in gene expression [3]. The mechanism for endogenous posttranscriptional regulation through miRNA was subsequently elucidated, and it was discovered that delivery of double-stranded RNA (dsRNA) achieved more potent gene silencing than ODNs [4,5]. Short hairpin RNA (shRNA), which more closely mimics the structure of endogenous miRNA, has also been delivered directly or via encoding plasmids [6]. In both cases, the enzyme Dicer cleaves the larger precursor/transcript to form small interfering RNA (siRNA), which is a 19−21 base pair, dsRNA [7]. siRNA can also be delivered directly to cells as a short dsRNA. The activated RNA-induced silencing complex (RISC) mediates recognition and enzymatic degradation of mRNA that is complementary to the siRNA antisense/guide strand [5].

Targeted exon skipping has been used to restore function of diseased proteins by masking splice motifs and removing out-of-frame exons or mutated pseudoexons

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from the mature mRNA. Exon skipping has been applied clinically for Duchenne muscular dystrophy and spinal muscular atrophy with the FDA approval of eteplirsen and nusinersen [8,9].

miRNAs are a class of noncoding RNAs that negatively regulate translation of mRNA. Single-miRNA molecules can regulate hundreds of genes and can act as regulators of entire gene networks. miRNAs are often dysregulated in local pathologies, and as a result, miRNA inhibition has been pursued to improve tissue regeneration [10].

Genome editing has received increasing attention with multiple platforms entering clinical trials for a variety of indications. Notably, zinc-finger nucleases have entered clinical trials for therapeutic cell therapy applications and for the first-in-man genome-editing clinical trial for Hunter's syndrome (MPS II, NCT03041324). CRISPR/Cas9 is used ubiquitously in research and has entered clinical development in cancer immunotherapy (NCT03399448) and was recently granted IND status by the FDA for inherited blindness [11]. CRISPR/Cas9 is extremely versatile as the endonuclease Cas9 is guided to a gene of interest using a guide RNA (gRNA). Genome editing is under extensive preclinical investigation in numerous applications including tissue engineering. Further, nuclease-deactivated Cas9 can be fused with a range of proteins to impart transcriptional control, modify epigenetic states [12], and make targeted single-nucleotide changes with base editors [13].

Gene replacement, RNAi, and genome editing all face common intracellular delivery barriers to reach the cytoplasm or nucleus (Fig. 29.2). Therapies for stable gene expression, including shRNA, must localize to the cell nucleus, while RNAi activity of siRNA depends upon delivery to the cytoplasm, where the RISC machinery is located. Nonviral approaches, which offer a potential safety margin, typically involve formulation with synthetic reagents such as polymers or lipids; however, these synthetic formulations are typically less efficient at triggering cell uptake and navigating subsequent intracellular delivery barriers. Recent advances in viral technologies have made these vectors safer than previous generations, and there are numerous ongoing trials for clinical applications of viral technologies. At the same time, high-throughput screening and other advanced synthetic approaches are yielding more efficient nonviral carriers than ever before, highlighted by the recent successful

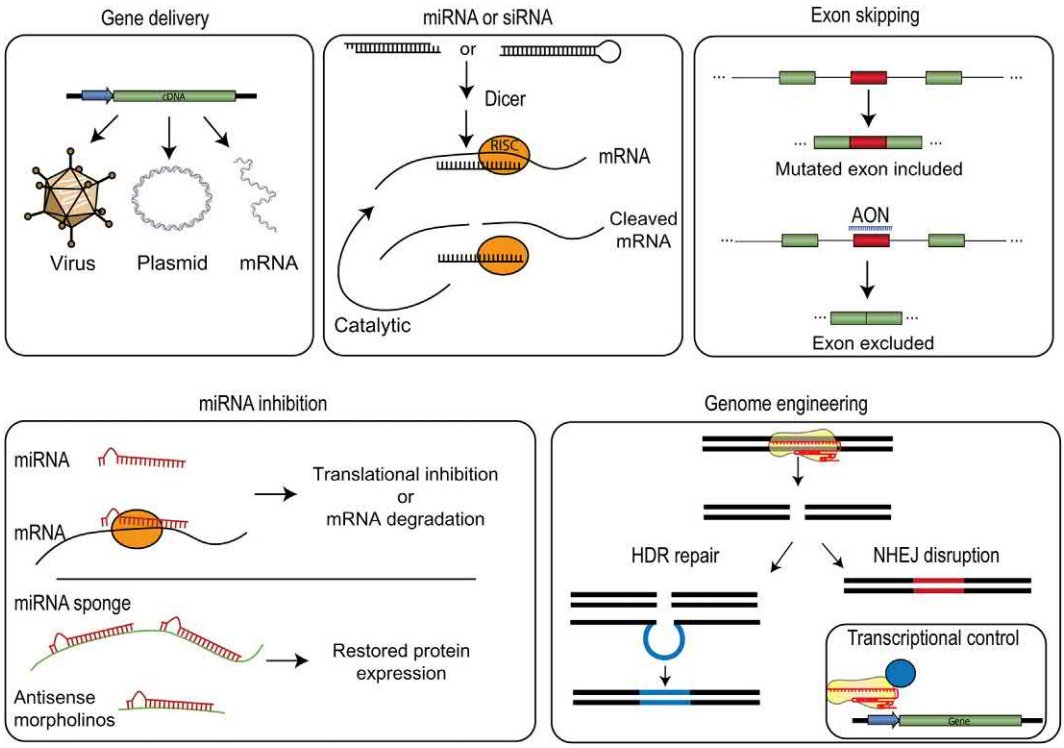

FIGURE 29.1 Gene delivery approaches under investigation. (A) Gene delivery is accomplished by packaging a desired transgene cDNA into a virus, a plasmid or a messenger RNA. (B) RNAi is accomplished by provided a siRNA or a short hairpin RNA to catalyze mRNA degradation. (C) Exon skipping is performed by provided antisense oligonucleotides that mask splice motifs removing target exons from the mature mRNA. (D) miRNA inhibition is performed by providing miRNA decoys or miRNA sponges that prevent translational inhibition. (E) Genome engineering can be accomplished by homology directed repair to precisely repair a gene, NHEJ to disrupt or remove segments of a gene, or to engineer transcriptional activators or repressors. *NHEJ,* non-homologous end joining; *RNAi*, RNA interference; *siRNA*, small interfering RNA.

RNAi product patisiran [2]. Optimized nonviral carriers are being applied broadly including genome engineering displaying high efficiency in CRISPR/Cas9-mediated in vivo hepatocyte editing [14]. Sections "Viral nucleic acid delivery" and "Nonviral nucleic acid delivery" survey the latest technologies for both nonviral and viral approaches to nucleic acid delivery, and subsequent sections address tissue engineering applications and recent clinical trial results.

Biodistribution, targeting, uptake, and trafficking

For successful gene therapy in vivo, viruses or nonviral DNA/RNA carriers have three main delivery barriers that must be overcome (Fig. 29.2):

1. efficient biodistribution to the desired site of action (i.e., tumor or site of tissue damage/dysfunction),
2. internalization of the cargo by the targeted cell type, and
3. trafficking to the subcellular compartment where the cargo will be active (i.e., cytoplasm for siRNA or nucleus for plasmid or genome editing).

Viruses have evolved to efficiently overcome the latter two barriers but achieving efficient access to the desired target cells is still a challenge. The successful design of synthetic delivery systems requires consideration of all three of these barriers and a thorough understanding of the mechanisms that dictate the interactions of delivery systems with target cells.

Tissue biodistribution/targeting

The most straightforward approach to site-specific delivery is local application, such as inhalation of dispersed solutions into the lungs to treat cystic fibrosis, or direct injection into the affected tissue, for example, subretinal injection for inherited blindness. In contrast, systemic delivery systems should be designed with functionalities that enhance the accumulation (biodistribution) of the therapeutic in the target tissue, optimize the kinetics of retention of the cargo within the target site, mediate specific binding to and uptake by a target cell population, and increase trafficking of the cargo to the desired intracellular compartment. These functions are often achieved through optimization of physicochemical properties to preferentially enhance nonspecific accumulation at defined sites and/or inclusion of ligands that target cell receptors uniquely expressed or overexpressed by the target cell type.

Key characteristics for intravenously delivered drug carriers are avoidance of rapid renal clearance or blood clearance by the reticuloendothelial system and preferential accumulation and retention at the site of disease. Long circulation half-lives are vital for optimal performance of delivery systems designed to passively accumulate in target tissues. In some cases, drug carriers with long circulation times can extravasate through abnormally large fenestrations of the cancer vasculature and nonspecifically accumulate in tumor tissues through the enhanced permeation and retention (EPR) effect [15]. The EPR effect occurs in animal models because poorly formed blood vessels in tumors allow leakage of nanomedicines into the tissue, with poorly formed lymphatic drainage contributing to the accumulation. Many cancer-targeted nanomedicines have been designed on this basis. Utilization of EPR effect has also been demonstrated for sites of tissue inflammation, including osteoarthritis [16]. However, It is important to note that the EPR effect has come under scrutiny for cancer therapy owing to variability of efficacy in clinical trials and as a result should be

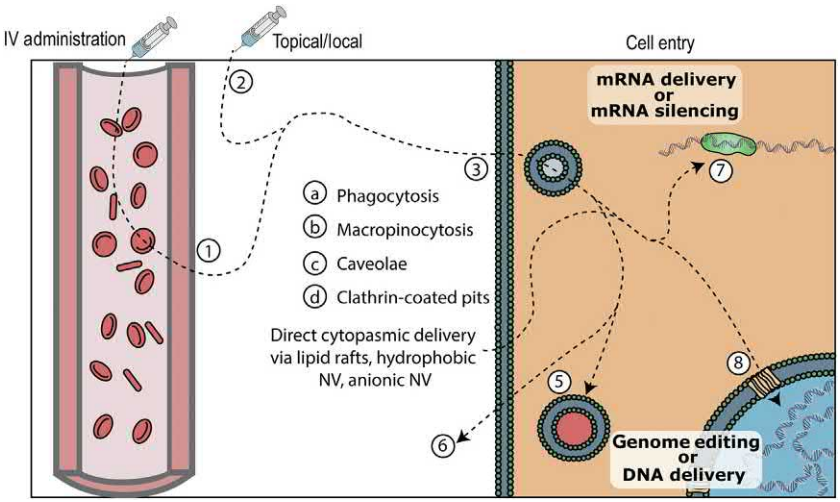

FIGURE 29.2 Gene delivery vehicles must navigate difficult delivery barriers. (1) IV administered vehicles must navigate the circulation and extravasate into the target tissue. (2) Local delivery can bypass the vascular barrier. (3) Particles must be internalized by cells through one of a number of mechanisms. (4) Particles are trafficked through the cytoplasm and must avoid (5) degradation in lysosomes and (6) exocytosis. After endosome escape, (7) mRNA and siRNAs must be delivered to the cytoplasm for activity while (8) DNA and genome editing machinery must be delivered to the nucleus for activity. *siRNA*, small interfering RNA.

evaluated on a case-by-case basis (see Refs. [17,18] for more discussion).

Optimization of physicochemical properties such as surface charge, size, shape, and mechanical properties are major design considerations for achieving the desired biodistribution of nonviral delivery systems [19,20]. Traditionally, nanocarriers have been synthesized using bottom-up synthetic approaches to form NPs such as micelles, dendrimers, and liposomes that have been predominantly limited to formation of spherical structures. For such structures, it has been hypothesized that NPs in the size range from approximately 10−200 nm may be ideal. Structures smaller than 10 nm suffer from rapid renal filtration and systemic clearance and larger structures will have decreased tissue penetration following extravasation [21]. One study administered 80−240 nm spherical NPs and showed the smallest NPs had longer circulation times, decreased hepatic clearance, and increased tumor accumulation [22] and another study showed that the biodistribution of NPs changed in response to systemic inflammation [23]. It may be desirable to design gene delivery vectors to be as small as possible, as long as they are above the size cutoff for renal filtration. However, NP assemblies larger than the renal filtration limit may still be cleared by renal filtration by disassembly at the glomerular basement membrane in the kidneys, especially in the context of the polycationic materials used for gene delivery [24,25]. It may be possible to stabilize the nanomaterial to avoid renal disassembly [26] or adhere to a long-circulating molecule like albumin [27].

In addition to size, the shape of nonviral vectors has a significant effect on behavior for in vivo systemic delivery applications; for example, rod-shaped filomicelles have 10 times longer circulation times relative to spherical micelles [28]. Innovative techniques have been developed in labs such as those of Mitragotri [29,30], DeSimone [30], and Roy et al. [31] that enable nanofabrication of particles with arbitrarily defined size and shape. Decuzzi et al. have contributed computational modeling and in vivo experimental data showing a significant effect of particle shape on biodistribution and ability to achieve vascular targeting [32]. They also demonstrated that shape-defined, discoidal, porous silicon nanovectors accumulate up to five times more in tumor tissues than spherical particles with similar diameters, and these shape-defined particles have also been effectively used for two-stage delivery of siRNA [33]. Decuzzi et al. has further demonstrated polymeric deformable nanoconstructs that can pass through small fenestrations, have high circulation time, and avoid clearance through the mononuclear phagocytic system [34]. In the case of micron-sized particles, stiffness of particles has been shown to have a significant impact, with stiffer particles rapidly distributed to the lungs, and those with a lower elastic modulus preferentially sequestered in the spleen [35].

Surface chemistry, in particular surface charge, can be tuned to act synergistically with physical properties such as size and shape in order to engineer optimal delivery systems. For example, neutrally charged NPs generally have lower rates of opsonization [36], and cationic NPs with superficial amines produce the highest levels of complement activation [37]. Xiao et al. applied a micellar NP (∼20 nM) to study the systemic effect of surface charge on biodistribution of a series of seven NPs with zeta potential ranging from −27 to +37 mV in tumor-bearing mice [38]. The authors determined that a "slight" negative charge (in their case, −8.5 mV zeta potential) was ideal for tumor accumulation and that "highly" positively or negatively charged NPs were preferentially cleared by macrophages (Kupffer cells) in the liver. Arvizo et al. [39] produced similar results using gold NPs, and they found that neutral or zwitterionic surfaces (−1.1 or −2 mV, respectively) produced longer circulation times and better tumor biodistribution than highly negative (−38 mV) or positive (+24 mV) NPs.

Most nonviral nucleic acid delivery systems involve formulation with an excess of cationic biomaterials in order to tightly package the polyanionic nucleic acid cargo into nano-sized particles. Unfortunately, the presence of surface-exposed cationic materials is not amenable to achieving the optimal, "slightly negative" negative surface charge desirable for systemically delivered nonviral vectors. Attachment of poly(ethylene glycol) (PEG), or PEGylation, is a popular approach for giving cationic carriers more stealth by shielding nonspecific cell interactions, reducing reticuloendothelial system clearance, and extending the blood circulation half-lives [40]. There are several fundamental properties of PEG that make it desirable for systemic delivery applications. It is a flexible, hydrophilic, and nontoxic polymer that reduces opsonization and nonspecific interaction with blood cells. It can reduce the access of degradative enzymes to drug cargo and provide increased steric bulk, which minimizes rapid renal clearance. The desirable performance of PEG is well documented in these regards, and PEG polymers are incorporated into therapeutic proteins and liposomal drug nanocarriers currently in the clinic. With the ubiquitous use of PEG, strategies to avoid immune recognition of PEG have emerged, including diversifying the structure of PEG in drug conjugates [41] and alternatives to PEG including zwitterionic phosphorylcholine−based polymers [42]. Zwitterionic poly-carboxybetaine conjugates have also been shown to have reduced immunogenicity compared to PEG even when conjugated to immunostimulatory proteins [43].

For gene therapy, one risk is that the inertness achieved by PEGylation can mask underlying

functionalities necessary to trigger cell uptake and/or endosomolytic activity of nonviral delivery systems. Interestingly, polyzwitterion nanocarriers appear to suffer less from the "PEG dilemma," meaning that large molecular weight zwitterionic polymers can be used that both reduce protein NP surface adsorption and increase circulation time, while maintaining favorable cell interaction/internalization properties [42]. The most common strategy for increasing internalization of PEGylated nanocarriers is incorporation of ligands, antibodies, or peptides that bind to specific cell receptors that internalize drug carriers and ferry them into the cell. Several promising approaches are also under development for attaching PEG by linkages that are reversible when exposed to signals uniquely present in the target tissue. For example, one can use attachment chemistries that are labile when exposed to tumor microenvironmental hallmarks, such as mild acidity and high matrix metalloproteinase (MMP) activity, in order to trigger shedding of PEG from the nanocarrier surface [44–46].

Conjugation of cell receptor ligands to the surface of nonviral carriers is one of the most studied preclinical approaches for enhancing localization, retention, and bioactivity in specific target tissues. Targeted delivery can potentially increase bioavailability of the therapeutic agent at its site of action and concurrently reduce off-site effects. Significant progress has been made in actively targeting unique markers on the vascular endothelium in specific tissues. For example, phage display technology has been instrumental in identifying targeting peptides that bind to specific, vascular "zip codes," and peptides identified using this technique present a potentially promising strategy for enhancing tissue-specific accumulation of nonviral delivery systems [47]. In order to achieve delivery into the tissue, transcytosis must occur across the vascular endothelium or, for the brain, the blood–brain barrier (BBB). Recently, systemic strategies to increase drug penetration into the brain have been developed, including temporary disruption of the BBB, chemical modification of available therapeutic substances, utilization of endogenous transport systems, and employment of lipid-based delivery systems [48].

Many other targeting approaches increase binding to cells at the target site once extravasation from the vasculature has occurred. For these approaches, biodistribution is primarily dictated by the physicochemical properties of the vector, and the targeting moiety affects retention and/or bioactivity at the site. Active binding to extravascular cells in the target site can enhance local retention through decreased diffusion or clearance from the tissue, and this prolonged residence time and/or targeting to actively internalized receptors can improve bioactivity. For example, cell-surface receptors are commonly targeted, because they have increased expression on a variety of cancer types leading to internalization through receptor-mediated endocytosis [49]. While there has been some clinical evaluation of targeted nonviral vectors [50], a variety of techniques are being actively pursued to develop targeting moieties based on natural ligands, antibodies or their fragments, peptides, aptamers, sugars, and small molecules. Furthermore, screening techniques such as peptide phage display are aiding in the discovery of new tissue-specific drug targets. Though they will not be reviewed in detail here, it should also be mentioned that several methods have also been studied for externally stimulated tissue targeting and drug release in response to stimuli such as light, ultrasound, ionizing radiation, or magnetic force [51].

Cellular uptake and intracellular trafficking

Once gene delivery systems have reached the target tissue, their route of internalization and trafficking to the desired subcellular compartment is paramount to achieving optimal bioactivity. To a large degree the mode of internalization dictates the subsequent route of trafficking within the cell. Thus an understanding of intracellular uptake and trafficking facilitates the design of better and more efficient gene delivery systems. Both experimental and simulation-based approaches can aide in the development of strategies for cellular internalization [52]. For additional information, the reader is referred to other reviews on nanovehicular uptake and trafficking [53,54].

Endocytosis is a generalized terminology used to describe cellular internalization. Endocytosis of nonviral vectors is the result of colocalization with the external side of the cell membrane (via receptors or nonspecifically) followed by formation of invaginations in the cell's plasma membrane that buds off inside the cell, to form lipid bilayer-enclosed vesicles that sequester the internalized cargo. Three kinetic modes of endocytosis can be defined: fluid-phase, adsorptive, and receptor-mediated endocytosis [55]. Fluid-phase endocytosis refers to the bulk uptake of solutes in exact proportion to their concentration in the extracellular fluid. This is a low-efficiency, nonspecific process. In contrast, receptor-mediated endocytosis involves internalization of macromolecules that are bound to and concentrated at the cell surface before internalization. In adsorptive endocytosis, molecules preferentially interact with nonspecific binding sites (e.g., electrostatic interaction of cationic vectors with anionic heparin sulfate proteoglycans). Internalized endocytotic vesicles subsequently undergo a complex series of fusion events that direct the internalized cargo to other intracellular compartments.

Endocytosis occurs by multiple mechanisms that fall into two broad categories: phagocytosis, or cell ingestion (of large particles), and pinocytosis, or cell drinking (of fluid and solutes) [56]. Phagocytosis is typically restricted

to specialized mammalian immune cells (dendritic, macrophages, monocytes, and neutrophils). Distinct subcategories of pinocytosis will be discussed further below, including macropinocytosis (MPC), clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CavME), and lipid raft-mediated endocytosis (LRME). Transcytosis and exocytosis, though they will not be discussed at length here, are "competing" cellular pathways that involve passage directly through the cell and expulsion of intracellular material into the external environment, respectively. A summary of different mechanisms is presented in Table 29.1.

MPC is generally considered to be a nonspecific process in which cells take up large volumes of extracellular fluids and solutes. MPC is characterized by membrane ruffling and formation of protrusions that ultimately produce large endocytic vesicles called macropinosomes ($>1\,\mu m$ in size, up to $5\,\mu m$), which sample large volumes of the extracellular milieu. The macropinocytotic process is constitutive in specialized cells (macrophages and dendritic cells) and in some tumors, and greater internalization may be achieved by nonspecific binding of solutes to the cell membrane (adsorptive pinocytosis). Although the internal pH of macropinosomes decreases, they do not fuse into lysosomes. This pathway provides some advantageous aspects, such as the increased uptake of particles and macromolecules, the avoidance of lysosomal degradation, and the ease of payload escape from macropinosomes due to their relatively leaky nature.

CME constitutively internalizes cell membrane sites that contain concentrated receptor–ligand complexes to form clathrin-coated vesicles (size $\sim 100-150$ nm), and CME can provide efficient cell uptake of essential nutrients, antigens, growth factors, and pathogens. Since receptors are differentially expressed in various cell types and tissues, receptor-mediated endocytosis via clathrin-coated pits provides a potential strategy for cell- and tissue-specific delivery of nonviral gene vectors. Molecules entering via this pathway experience a drop to approximately pH 6 in the early endosome, with a further reduction to approximately pH 5 during progression to late endosomes and lysosomes [57]. While the initial events in the endocytosis of the receptor–ligand complex are similar for most systems, the processing of the ligand can differ, depending on both receptor and cell type. Following CME, ligands and receptors are sorted to their appropriate cellular destinations, such as lysosomes, the Golgi apparatus, the nucleus, or back to the cell-surface membrane. In the conventional model the internalized ligands are degraded in the acidic endo-lysosomal compartments while the receptor is recycled back to the plasma membrane.

CavME is mediated by flask-shaped invaginations in the cell membrane called caveolae (50–60 nm in size) that are particularly abundant on endothelial cells. Caveolae are cholesterol and sphingolipid-rich microdomains of the plasma membrane, and they are concentrated "hot spots" for a diversity of signaling molecules and

TABLE 29.1 Efficiency of nanoparticle entry via multiple portals as differentiated by cargo chemistry/size[a] and cell type.

Phagocytosis		Pinocytosis				
		Macropinocytosis (fluid phase)	CME	Caveolae-mediated rafts	Lipid rafts	Clathrin- and caveolin-independent
Vehicle size	1–10 μm[b]	1–5 μm[b]	<150 nm	<60 nm	40–60 nm	200–300 nm
Cell types	Dendritic, macrophages, monocytes	Many cells	Many cells	Differentiated endothelial cells, apipocytes, epithelial, and muscle cells	Lymphocytes, cancer cells, rodent macrophages	Specialized cells, endothelial cells, and others
Efficiency of uptake[c]	+++++ for specialized cells	+++	+++++	+	+	++++

CME, Clathrin-mediated endocytosis.
[a]*Typically, cargo chemistry determines a size range due to technological limitations (e.g., dendrimer-based and micellar-based nano-vehicles are limited to rather small sizes).*
[b]*Phagocytosis and macropinocytosis most likely do not contribute to the nanovehicular uptake.*
[c]*The efficiency of uptake may depend on nanoparticle type and chemistry. The proportion between different uptake mechanisms may vary. Estimated on scale of 1–5, 5 being the highest.*

membrane transporters. A unique aspect of CavME is that it is a nonacidic and nondigestive route of internalization that bypasses lysosomes and thus may be an advantageous route for drug delivery. However, caveolae are internalized slowly and their fluid-phase volume is small; this results in low uptake capacity for external cargo. Although endothelial caveolae constitute 10%−20% of the cell surface [56], it is unlikely that they contribute significantly to constitutive endocytosis.

LRME-independent uptake is an alternative pathway that can be operative for anionic and neutral-lipid liposomes, solid-lipid NPs, and hydrophobic (i.e., polystyrene) NPs. Lipid rafts are cholesterol- and sphingolipid-rich 40−60 nm microdomains that are characteristic of cells that lack caveolin and caveolae. This uptake mechanism is likely mediated through scavenger receptors that are involved in the uptake of both lipophilic and anionic cargo [58]. For example, scavenger receptor class B (SRB1; CD36 superfamily of proteins), which is expressed on mature macrophages, binds to a diversity of polyanionic protein, polyribonucleotide, polysaccharide, and lipid ligands. The involvement of SRB1 in liposome uptake has been further supported by the competitive inhibition of hepatic cell uptake of neutral phospholipid/cholesterol liposomes by the strong polyanion polyinosinic acid [59]. LRME bypasses lysosomes and is facilitated by the cellular transmembrane potential. This is a unique feature of CavME, requires an energy input.

Physicochemical properties of NPs play a significant role on the level of uptake and the mode of transport into cells. Numerous studies have shown that surface charge has a significant impact on cellular internalization of nanocarriers [60]. Size has also long been known to affect uptake, with several studies concluding that NPs in the 100−200 nm range possess the best properties for cellular uptake [61]. Great strides have been made in understanding the interplay between size and shape on cell internalization. Using polystyrene particles of various sizes and shapes, Champion and Mitragotri studied phagocytosis by alveolar macrophages, and they were some of the first to report on the importance of shape rather than just size [62]. In particular, the local particle shape at the cell-particle interface was found to dictate macrophage phagocytosis versus spreading of particles (Fig. 29.3A). DeSimone et al. tested uptake in nonphagocytic HeLa cells for a series of particles with varying sizes and shapes that were fabricated using a lithographic method called PRINT (particle replication in nonwetting templates; Fig. 29.3B) [63]. They found that the internalization kinetics of the NPs by HeLa cells depended on aspect ratio and particle size and/or volume. The most striking finding was that rod-like particles were internalized more rapidly, with cylindrical particles of 150 nm diameter and a 3:1 aspect ratio showing the most rapid uptake in their study. Their data also indicated that the PRINT particles used a combination of the different internalization pathways described above to enter the cells, suggesting that size and shape alone cannot be utilized to fully define the mechanism of uptake and subsequent intracellular trafficking.

In addition to optimizing their physical properties, nanovectors can be endowed with a variety of other specialized constituents that dictate their mode of cellular internalization, either by binding to specific internalizing receptors or by directing traffic to specific intracellular compartments such as the cytoplasm and nucleus. There are several examples of amine-containing polymers that buffer endosome acidification and lead to endosome disruption through the osmotic proton sponge mechanism. Poly(alkylacrylic acids) are another class of polymers that can mediate endosomolysis, but they act through an active membrane disruption mechanism. In this case, protonation of carboxylates within acidic environments transitions the polymers from an anionic, hydrophilic state into an uncharged, hydrophobic, and more compact state that is membrane interactive/disruptive [64]. NPs that show a transition in their surface charge from anionic (at pH 7) to cationic in the acidic endosome (pH 4−5) have can thereby escape from endosomal compartments [65].

For plasmid gene delivery, trafficking to the nucleus is of particular significance. As discussed in section "Nonviral nucleic acid delivery," polymers and lipids are the two main classes of biomaterials used to formulate nonviral vectors, and choice of vector can affect, among other things, the mode of cell internalization. For example, the uptake of cationic lipids proceeds mainly by the clathrin-dependent pathway, but internalization of polymer-based systems is more dependent on the polymer and the cell type. Polymer-based delivery systems can simultaneously proceed by both clathrin-dependent and clathrin-independent pathways in the same cells [66]. Separately blocking either the clathrin- or caveolae-dependent pathway does not dramatically affect quantity of vector uptake, but blockage of caveolae-mediated uptake significantly abrogates gene expression, suggesting this route of uptake plays a significant role in intracellular trafficking to the nucleus [66,67]. In agreement with this result, Sullivan et al. have shown that use of histone H3 tail peptides that predispose trafficking of polyplexes to caveolar pathways can be used to enhance plasmid transfection [68]. Their follow-up work has provided mechanistic evidence that caveolar polyplexes are trafficked to the nucleus through a retrograde Golgi-to-ER (endoplasmic reticulum) pathway [68]. Other mediators of intracellular trafficking have also been successfully codelivered to modulate NP intracellular trafficking. For example, fusogenic, endosomolytic peptides or nuclear localization signals can be attached to NPs to enhance targeting to the

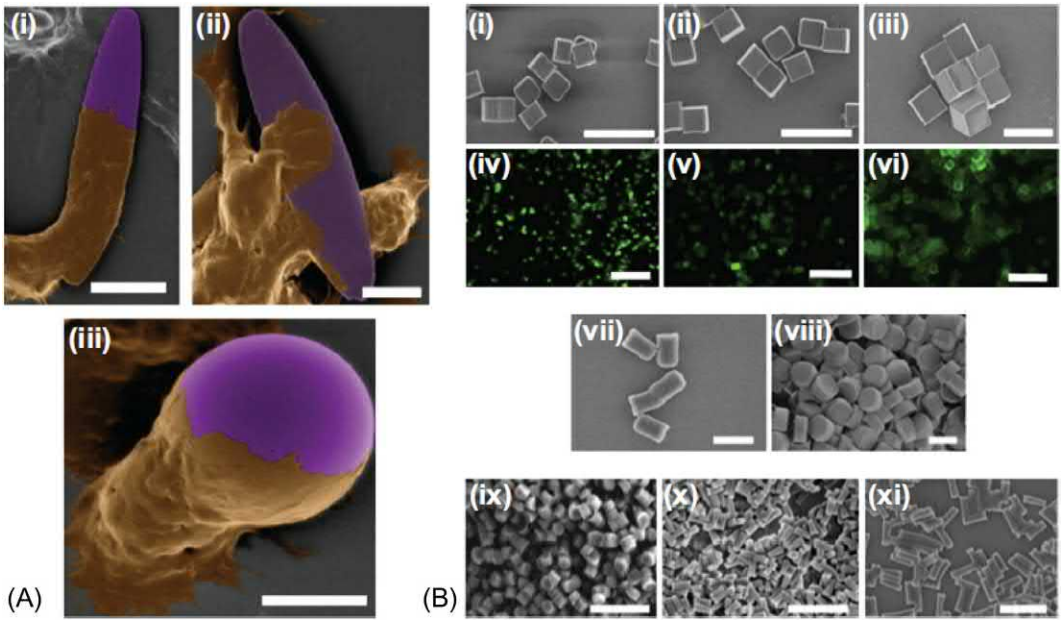

FIGURE 29.3 Nanoparticulate shape variation. (A) Scanning electron microscopy images provided evidence that particle internalization is dependent on local particle shape from the perspective of the phagocyte (cells and particles were colored brown and purple, respectively). Phagocytosis progressed further when the cell approached the end of the opsonized elliptical particle relative to when contact was initiated on the flatter, more elongated side of the particle. Phagocytosis progressed further when a smaller particle dimension was approached by the cells, that is, as shown for the smaller spherical particle in panel (iii). Scale bar = 10 μm is subpanel (i) and 5 μm in subpanels (ii)−(iii) [43]. (B) Panel of micrographs demonstrating PRINT particles varying in both size and shape. Subpanels (i)−(iii) show scanning electron micrographs of cubic particles of size 2, 3, and 5 μm, respectively, and subpanels (iv)−(vi) show fluorescence micrographs of the same cubic particles. Subpanels (vii) and (viii) show scanning electron micrographs of cylindrical microparticles of 1 μm in height with diameters of 0.5 and 1 μm, respectively. Subpanels (ix)−(xi) show scanning electron micrographs of (ix) 200 nm diameter and 200 nm height (x) 100 nm diameter and 300 nm height and (xi) 150 nm diameter and 450 nm height. Scale bars = 20 μm for (i)−(vi) and 1 μm for (vii)−(xi) [44].

nucleus. Recently, it has also been shown that treatment with histone deacetylase inhibitors can be used to liberate polyplexes from sequestration at the perinuclear recycling compartment/microtubule organizing center and to thereby increase transgene expression by up to 40-fold [69].

Viral nucleic acid delivery

Introduction to viral gene therapy

A virus is a biological NP consisting exclusively of components including proteins, lipids, carbohydrates, and nucleic acids. Each virus carries a viral genome that encodes the structural and functional elements of the infectious particle, but it does not encode the necessary machinery for transcription, translation, and genome replication. Therefore viruses infect living cells and can only replicate inside these hosts by hijacking native cellular transcription and translation processes.

Viruses have evolved to be extremely effective at infecting and replicating within host cells. Therefore many of the challenges of nonviral gene delivery, such as cellular uptake, trafficking, and nuclear import, are readily addressed by the innate biological properties of viral vectors. To take advantage of these properties, researchers have reengineered natural viral vectors by removing all pathogenic components and signals necessary for virus replication within infected cells and replacing them with therapeutic genetic sequences. This approach has been widely successful in engineering vectors for efficient gene delivery in research but has encountered severe hurdles en route to clinical efficacy.

The first gene therapy clinical trial in 1990 used a retroviral vector for gene delivery to T cells of patients with severe combined immunodeficiency [70]. After 30 years, there have been over 2000 new clinical gene therapy clinical trials worldwide with over 200 initiated in both 2017 and 2018. Over 66% of these trials have used viral vectors for gene delivery [71]. The first gene therapy approved for clinical use was an adenoviral p53 vector for cancer treatment in China in 2003 [72]. In 2012 a landmark gene therapy was approved in Europe consisting of an adeno-associated virus (AAV) encoding a gene to treat a rare genetic disorder known as lipoprotein lipase deficiency (LPLD) [73], although it has since been withdrawn from the market. In 2018 several new approvals and promising

clinical trial results indicate the potential for viral gene therapy, but the delay in clinical success also underscores the challenges in viral vector engineering.

The viral gene therapy field encountered severe complications before reaching this more recent emergence of positive results. In 1999 a patient named Jesse Gelsinger was treated in a clinical trial with an adenoviral vector as a therapy for a genetic metabolic disease. He died 4 days after vector administration due to a severe inflammatory episode and multiorgan failure, which was later linked to an immune response against protein components of the virus [74]. One year later, another landmark event occurred when the first reports of successful gene therapy for curing a genetic disease, in this case a fatal immunodeficiency disorder, were reported [75]. In all, 20 children were treated with autologous hematopoietic stem cells transduced with retroviral vectors carrying a therapeutic transgene, and ultimately 17 of these patients were cured of the disease [76]. However, five of these cured patients later developed leukemia as a result of integration of the viral vector into the genome near to an oncogene, leading to oncogene activation [77]. One of these five patients died as a result of the leukemia. Due to these adverse events, viral gene therapy was the subject of social and scientific stigma in the early part of the 21st century. However, investigators have been able to identify and address the mechanistic basis of these adverse events, leading to improved viral vectors in numerous ongoing and successful clinical trials. Nonetheless, the fundamental source of these challenges is the complexity of viral vectors, each of which contains hundreds to thousands of copies of dozens of distinct biological macromolecules that perform complex functions. The complexity of viral biology is a primary motivating factor for engineering nonviral delivery vehicles comprising defined chemical components to achieve precise control over all elements of gene transfer and expression.

Although the clinical trial results discussed above are largely focused on rare hereditary disorders, the history and critical concerns of viral vector development are essential to understanding the potential of viral nucleic acid delivery for tissue engineering and regenerative medicine. Furthermore, new medical technologies such as gene delivery are often first developed for fatal diseases for which there is no other option, such as genetic disorders and cancer. The technology development and demonstration of safety and efficacy of gene transfer in these areas is directly translatable to applications in other fields, including tissue engineering.

Types of viral vectors

A variety of viral vectors have been employed to deliver genes to cells. Each approach has its own advantages and disadvantages, including packaging capacity, persistence of expression, immunogenicity, and nonspecific effects on the cellular genome [78,79].

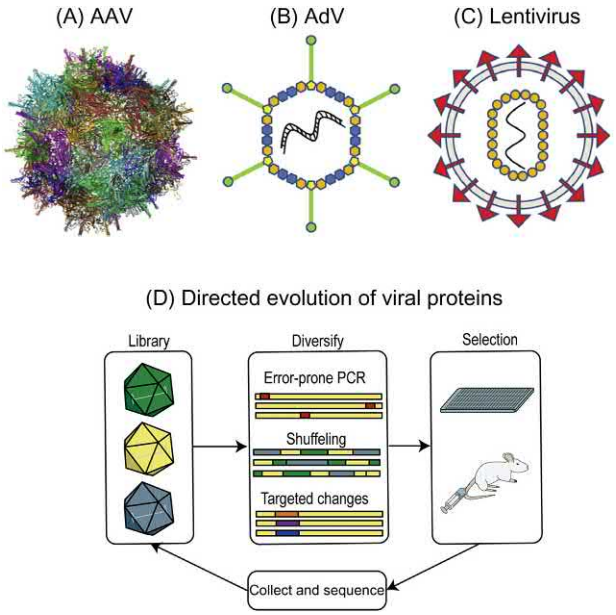

FIGURE 29.4 Representative schematics of viral structures and directed evolution of viral proteins. (A) Adeno-associated virus. The crystal structure of the AAV capsid proteins that encompass the vector and interact with cellular receptors is shown (MMDB ID 20256). Capsid monomers are represented by different colors. (B) Adenovirus. Adenoviral vectors consist of a capsid shell made up of proteins known as hexon (blue), penton (orange), and the penton base (yellow), to which the fiber protein (green) is attached. The double-stranded DNA viral genome is packaged inside the capsid. Other accessory proteins are not shown. (C) Retrovirus/Lentivirus. Retroviral and lentiviral vectors are encompassed with a lipid bilayer envelope (gray), which contains proteins that interact with cell surface receptors (red). Within the envelope, a capsid shell (orange) surrounds the single-stranded RNA genome. (D) Viral vectors can be improved by directed evolution to avoid neutralizing antibodies or find new tissue tropism. Several methods are available to diversify viral libraries including error-prone PCR, capsid shuffling, or targeted changes to the capsid structure. *AAV*, Adeno-associated virus.

Adenoviruses remain a commonly used therapeutic viral vector, largely due to applications in cancer therapy. An adenovirus consists of a double-stranded DNA genome and a protein shell, known as a capsid (Fig. 29.4A). The adenoviral particle binds to cells through fiber proteins in the capsid that recognize specific receptors on the cell surface, followed by endocytosis of the virus. Adenoviral vectors are engineered as replication deficient, exist extrachromosomally, and therefore are not copied with the cellular genome during cell division. As a result, gene expression is transient as the vector is degraded or diluted out during cell replication. The adenovirus has an outstanding ability to escape endosomes, enter the cytoplasm, and pass through the nuclear membrane pore. Despite extensive investigation of dozens of

viral vectors for cancer treatment and promising results in clinical trials, the FDA (Food and Drug Administration) has not approved any adenovirus-based therapeutics to date. Because they cause significant immunogenicity, adenoviral vectors can only be delivered once to a patient. Therefore adenoviruses are not an optimal choice for chronic conditions but can be very useful for modifying cells ex vivo. The primary advantage of the adenovirus is its ability to achieve very high viral titers ($>10^{12}$/mL). Unlike unstable RNA viruses with lipid bilayers, including retroviruses and lentiviruses, adenoviruses can easily be concentrated and these vectors can infect dividing as well as nondividing cells with high efficiency. This is a major advantage for some applications, including in vivo gene transfer to tissues such as the lung, where cell division is infrequent.

AAV are small viruses with single-stranded DNA genomes and a protein capsid (Fig. 29.4B). Unlike other viral vectors developed for gene therapy, wild-type AAV is not known to cause any human disease. The main disadvantages of AAV are that the vector can efficiently package only small transgene cassettes (up to 4.7 kb), the capsid proteins can be immunogenic, and the preparation of the recombinant vector is complex. Nonetheless, AAV is becoming the preferred vector in the gene therapy field [80,81]. This is because like adenovirus, AAV can be prepared to high concentrations and is relatively stable in storage. In addition, recombinant AAV does not efficiently integrate into the genome, and, therefore, there is little risk of disruption of native genes. In contrast to adenoviruses, the AAV vector is particularly stable within the nucleus and can exist with high levels of gene expression for months or years [82–85]. As discussed in more detail next, many serotypes of AAV exist, and the capsid proteins are exceptionally flexible for being reengineered to target new cell types. AAV can also be engineered for tissue-specific targeting following systemic delivery [86].

Retroviral and lentiviral vectors contain a single-stranded RNA genome, a protein capsid, and a lipid bilayer, known as the envelope, that also contains transmembrane proteins (Fig. 29.4C). The vectors are able to efficiently carry about 8–10 kb of transgenic material. A disadvantage of these vectors relative to adenoviruses and AAVs is that the RNA genome and lipid bilayer make them comparably unstable, with a half-life of less than 12 hours at 37°C. This also creates challenges for storage, and the titers of these viruses are relatively low (10^6–10^7 particles per mL), although they can be concentrated to $>10^9$/mL. The major advantages of retroviral and lentiviral gene transfer are that, in contrast to nonviral systems, recombinant viruses are capable of transferring genes to a wide range of different primary cell types, and the genes are stably integrated into the chromosomal DNA. Therefore these vectors are ideal for applications requiring long-term gene expression. However, the integration of these viral vectors into the genome also has the potential to disrupt endogenous genes, such as critical oncogenes and tumor suppressors, as discussed earlier [87]. There are two ways in which this has been addressed. First, self-inactivating (SIN) vectors have been developed in which the strong viral promoters and viral splice sites have been disabled, and therefore the virus is less likely to alter the expression of nearby genes [88]. Second, integrase-deficient lentiviral vectors have been engineered that have the advantages of high-efficiency lentiviral transduction. However, these virions do not integrate into the genome, and they result in only transient gene expression [89]. It is becoming more common that lentivirus is preferred over retrovirus, as retroviral transduction is dependent on cell division, whereas lentiviral transduction is not [90].

Herpes simplex virus type-1 (HSV-1) is a human neurotropic virus used primarily as a vector for gene transfer to the nervous system, although the wild-type HSV-1 can infect and lyse other nonneuronal epithelial cells [91]. Because of its large genome size, up to 30–50 kbp of transgenic material can be packaged into recombinant HSV-1 vectors. At present, two major classes of HSV-1 vectors have been developed: replication-defective viruses and replication-conditional mutants. However, the ability of HSV and these mutant recombinants to establish lifelong latent infections raises concerns about the use of these vectors in humans. Efforts have been made to create HSV-1 amplicon vectors essentially free of HSV-1 helper virus, which might be a promising genetic vehicle for in vivo gene delivery.

Most commonly, viral vectors are used to deliver cDNAs of transgenes encoding proteins that will confer some therapeutic effect. In the context of tissue engineering, these transgenes may facilitate progenitor cell expansion, cell differentiation, tissue formation, and/or wound healing. However, viruses can also be used to deliver siRNAs to silence target gene expression [92]. In order to take advantage of the RNAi mechanisms of processing short dsRNAs by the RISC complex, viral vectors are designed to express shRNAs that mimic the structure of the naturally occurring miRNAs. Both AAV and lentiviral vectors are routinely used for shRNA-mediated gene knockdown. Other possible cargo for viral vectors includes aptamers, ribozymes [93], and genome-editing technologies [94]. Viral particles have also been engineered for the transfer of proteins across the cell membrane [95].

Engineering viral vectors

The natural evolution of viruses has produced a wide variety of gene transfer vectors with innate capabilities

for overcoming many of the challenges of gene delivery to living cells, including recognition of cell-surface proteins, internalization, endosomal escape, trafficking to the nucleus and transfer across the nuclear membrane, and ultimately transcription of viral genes. However, these natural properties do not necessarily address all of the challenges for many applications of gene delivery for gene therapy, regenerative medicine, and tissue engineering. Additional challenges include virus purification, immunogenicity of viral proteins, proper virus localization following systemic delivery, targeting the virus to specific cell types, tissue-specific gene expression, and controlled release of viral vectors from biomaterials. To address these challenges, the biological properties of these vectors can often be reengineered via rational design. Alternatively, the complex properties of these systems may be more easily altered by high-throughput selection of large libraries of variants of viral particles (Fig. 29.4D) [96].

Viral particles predominantly interact with cells via the proteins on the surface of the virus. In the case of retroviruses and lentiviruses, these interactions occur through the proteins in the lipid envelope that comprise the outer surface of the particle, whereas for adenoviruses and AAVs, the capsid proteins encompass the particle [96]. Therefore exchanging these proteins with those of other serotypes, in a process known as pseudotyping, provides a means of changing the tropism of the virus, which is defined by the types of cells the virus will transduce. Modifying the capsid and envelope proteins can also be used to alter the recognition of the viral particles by the immune system. For example, pseudotyping adenoviral vectors with alternative capsid proteins led to increased transduction of human cell lines and primary cells, including transduction of intact human saphenous veins and human bone marrow stromal cells for seeding onto scaffolds for bone tissue engineering [97]. Adenoviral pseudotyping has also been used to evade antivector immunity [98]. The pseudotyping of retroviruses and lentiviruses is particularly straightforward, since the envelope proteins exist in the lipid bilayer and do not serve important structural roles, as the capsid proteins do [99]. This approach has been used to broaden virus tropism to facilitate transduction of many cell types [100] or to narrow tropism to limit transduction to a specific cell type, such as neural stem cells in the brain [101]. Pseudotyping these vectors can also enable the transduction of tissues that are otherwise resistant to gene transfer, such as the airway epithelium [102] and spinal cord [103]. AAV has been pseudotyped both by using capsid proteins from alternative serotypes, mixing capsids from two serotypes, and generating single chimeric capsid proteins with elements from multiple serotypes [96].

As an alternative to pseudotyping, which uses surface proteins from other virus serotypes, grafting targeting peptides into the viral genome can also functionalize the capsid and envelope proteins. For example, insertion of the Arg-Gly-Asp integrin-targeting peptide sequence into adenoviral vectors has directed viral transduction to cells expressing high levels of integrin receptors [104,105]. This approach has also been used to preferentially direct viral transduction to endothelial cells [106]. Alternatively, biotin-binding peptides [107], single-chain antibodies [108], and genetically encoded imaging agents [109,110] have been incorporated into adenoviral capsid proteins as general approaches for redirecting cell targeting or tracking viral localization. Similar strategies have been explored for AAV including targeting ligands [111], biomolecular conjugates [112], and protease-activatable targeting [113]. Single-chain antibodies have also been grafted into retroviral and lentiviral vectors to control targeting [114], though typically with a decrease in overall transduction efficiency [115]. Alternatively, growth factors and cytokines have been inserted into the envelope to target transduction to cells bearing the complementary receptor [116,117].

In many cases, our understanding of the structure−function relationships of the viral proteins is insufficient to allow rational design of highly active engineered proteins with new functions. In these circumstances a directed molecular evolution strategy may be useful. In this approach, large viral variants libraries allow for selection of variants with desirable properties [118]. For example, display of random peptide libraries on AAV and adenovirus have led to vectors with increased selectivity and/or transduction efficiency of endothelial cells [119] and myoblasts [120]. Random mutagenesis or DNA shuffling and selection of novel AAV capsids has led to new variants with beneficial immune evasion and purification properties [121,122], and this approach has enhanced the transduction of human airway models [123,124], specific neural cell types [125,126], and induced pluripotent stem cells [127]. Collectively, these technologies provide a means to design improved viral vectors for a variety of applications.

In contrast to genetic modification of the viral proteins, adaptors or chemical modifications can be used to redirect viral transduction. PEGylation can be used to mask viral vectors from the immune system, but in many cases, this approach will decrease overall transduction efficiencies [128]. In contrast, conjugation of the PEG group to a functional targeting moiety, such as an antibody, can lead to simultaneous protection of the vector and delivery to specific cell types in vivo, such as endothelial cells [129]. Other work has used bispecific adaptors that bind both the viral surface proteins and a particular cell-surface receptor [130,131]. The incorporation of antibody-binding domains or biotin molecules into viral capsids has led to a general approach in which

the virus can be retargeted to any cell type via an antibody linker targeted to a cell-surface epitope [132] or a fusion protein of streptavidin and a targeting ligand [133].

The methods described above are predominantly designed to control which cells a virus will transduce. Another approach to controlling gene expression is to use an engineered promoter that can be regulated in magnitude, time, space, and/or tissue type [134]. These approaches are applicable to both viral and nonviral systems. The most commonly used inducible gene expression system is based on the antibiotic tetracycline [135]. Examples of this system that are relevant to tissue engineering include its use to control gene expression for bone tissue engineering [136,137], treatment of arthritis [138], and control of drug or hormone production by engineered tissues or implanted cells in vivo [139], among others. Systems have also been developed to control gene expression in response to a variety of other chemical inducers, including ecdysone [140], rapamycin [141], and other antibiotics. Other systems to control gene expression of implanted cells with an exogenous operon have been based on skin lotions containing phloretin [142] and inhaled gases containing acetaldehyde [143].

In addition to chemical inducers, gene expression can also be controlled from promoters that are sensitive to hypoxia and temperature that have been used to trigger gene-mediated cardioprotection and angiogenesis [144,145]. More recently, optogenetic approaches have emerged in which genes are placed under the control of light-inducible proteins from plants or the eye [146–148]. Consequently, the magnitude, dynamics, and spatial patterning of gene expression can be controlled with illumination in vitro and in vivo. Engineered transcription factors can be designed for light-inducible transcriptional activation in vivo with a far-red illumination source. A recent report showed light-induced neuronal differentiation with a CRISPR-based, light-inducible transcription factor [149]. In the future, this approach may be used to control tissue formation as part of a regenerative medicine strategy. Finally, gene expression can be restricted to certain cell types by regulating transgenes with promoters that correspond to genes only expressed in particular tissues. This approach has been widely employed to create promoters that are specific to bone, cartilage, skeletal muscle, cardiac muscle, retina, and liver.

Nonviral nucleic acid delivery

Introduction to nonviral nucleic acid delivery

While viral delivery systems are equipped with inherent, evolved mechanisms for efficient intracellular delivery, nonviral nucleic acid delivery systems can offer a well-defined and potentially safer alternative that can be cheaply made on large scales. However, optimizing these systems to approach the efficiency of intracellular delivery achievable with viral vectors has remained elusive. A variety of synthetic systems have been sought for nonviral nucleic acid delivery, primarily based on polymers (synthetic and natural), lipids/liposomes, and inorganic NPs (Fig. 29.5). Significant progress has been made in the development of nonviral carriers that can overcome the

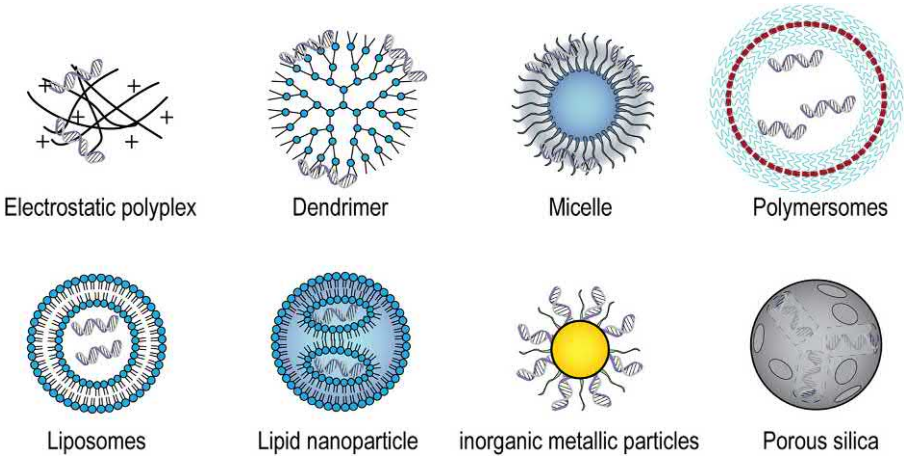

FIGURE 29.5 Nonviral gene delivery formulations. (A) Polymers are assembled into a range of nanosized structures. Electrostatic polyplexes formed by the condensation of positively charged polymers and negatively charged nucleic acid. Positively charged dendrimers electrostatically condense siRNA into polyplexes. Micelle structures have hydrophobicity in the core and typically have a positively charged corona to form complexes with nucleic acid. Polymersomes are formed from amphiphilic block copolymers forming an aqueous center. (B) Lipid materials are used to formulate siRNA and are the first approved delivery vehicle for an RNAi drug. Liposomes are formed from lipoid materials into a lipid bilayer with an aqueous center. Lipid nanoparticles are composed of a nanostructured core. (C) Inorganic particles are used as a scaffold for the construction of nanosized vehicles that often have additional imaging and therapeutic capabilities. Gold nanoparticles are often used in the delivery of siRNA. Porous silica nanoparticles have a large internal surface area, are biodegradable, and can be charge agnostic making them better suited for delivery peptide nucleic acids. *RNAi*, RNA interference; *siRNA*, small interfering RNA.

primary intracellular delivery barriers such as cellular internalization, escape from endo-lysosomal pathways, and trafficking to the desired intracellular space (i.e., cytoplasm for siRNA and nucleus for plasmid). Although the available design space for engineering of nonviral vectors is vast, and numerous preclinical studies have emerged in the last few years with exciting results, to date, there are two clinically approved, nonviral gene delivery product indicating the significant engineering challenge for nonviral gene delivery. The remainder of this section will provide a more thorough overview of nucleic acid modifications and polymer, lipid, and inorganic NP technologies that have been recently developed for nucleic acid therapeutics.

Oligonucleotide modifications

Nonviral delivery vehicles provide the opportunity to engineer the oligonucleotide chemistry to improve stability, nuclease resistance, activity, and immunogenic properties of delivered oligonucleotide cargo (Fig. 29.6A). For siRNAs, 2′-*O*-methyl modifications, 2′-Fluoro modifications, and a phosphorothioate backbone have shown improved properties in large animal studies and clinical trials [2]. Messenger RNA has been modified to avoid innate immune responses caused by ssRNA delivery [150,151]. One study found that 25% substitution of 2-thiouridine for uridine and 5-methylcytidine for cytidine avoided toll-like receptor recognition and led to protein production in mice [151]. Intravenous delivery of lipid NPs (LNPs) containing modified mRNA encoding vascular endothelial growth factor (VEGF)-A improved cardiac function in a mouse model of myocardial infarction [152]. Another study used modified mRNA encoding VEGF-A in patients with type 2 diabetes led to increased VEGF-A expression and improved blood flow [153]. For genome editing, modifications to the gRNA have been used to improve nuclease resistance and reduce innate immune response in vivo. In a recent report from Intellia Therapeutics, an LNP system delivering Cas9 mRNA and modified gRNA edited the mouse transthyretin gene in hepatocytes reducing serum protein levels by 97% [14]. Peptide nucleic acids (PNAs) are an engineered variant of nucleotides with a peptide backbone [154]. PNAs have been used to interfere with miRNAs [155] which have substantial roles in tissue engineering (reviewed here [10]). PNAs have been delivered to mouse hepatocytes with porous silicon NPs to interfere with miR-122 in mice leading to improved HDL cholesterol values [156]. PNAs can also be designed to form triplexes with genomic DNA to force strand invasion and induce DNA repair and precise genome editing (reviewed [157]). A recent study by the Glazer and Saltzman groups used PNAs in a mouse model of beta-thalassemia delivered by intravenous administration of PLGA NPs achieved 7% gene correction [158]. Follow-up work from the same groups showed an increase in gene correction up to 10% when administered in utero, which led to improved survival in mice up to 500 days post injection [159].

Conjugates

Direct conjugation of macromolecules can enhance the bioavailability of oligonucleotides (Fig. 29.6B). Molecular conjugates will likely comprise the next wave of approved siRNA products with several candidates in late clinical trials with one approved product (Givlaari). Most notably, *N*-acetylgalactosamine (GalNAc) conjugates have been used for efficient liver delivery of siRNAs by binding the asialoglycoprotein receptor on hepatocytes resulting in internalization and target gene silencing [160]. Other promising conjugates include the hydrophobic modifications palmitic acid [161,162], cholesterol [163,164], and diacyl lipid [165]. Lipid-modified siRNAs designed to bind to the serum protein albumin greatly improve in vivo circulation half-life and bioavailability and may have advantages over application of larger, conventional NPs for tumor targeting (see review Ref. [27]). For example, a diacyl lipid−modified siRNA shows improved circulation time and tumor accumulation in a patient xenograft model of triple-negative breast cancer overcoming limitations of the absence of the EPR effect in many tumor models [165].

Synthetic polymers

A variety of natural and synthetic polymers have been developed for nucleic acid delivery systems. This includes standard synthetic polycations, such as polyethylenimene (PEI), poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA), and cationic dendrimers, biodegradable synthetic polycations such as poly(beta-amino esters) (PBAE), glycopolymers, and peptide/protein-based polymers.

The earliest polymeric approaches to nonviral gene therapy employed cationic, amine-rich polymers such as PEI, PDMAEMA, and the cationic poly(amido amine) (PAMAM) dendrimers (Fig. 29.7A). PEI is commonly used in both in its linear and branched forms for DNA plasmid delivery, whereas PDMAEMA is typically utilized as a linear homopolymer or as a diblock polymer with PEG or other compositions. Dendrimers such as PAMAM have more complex “tree-like” architectures that form spherical, monodisperse macromolecules. These branched structures are synthesized either from the central core toward the periphery (divergent synthesis) or starting

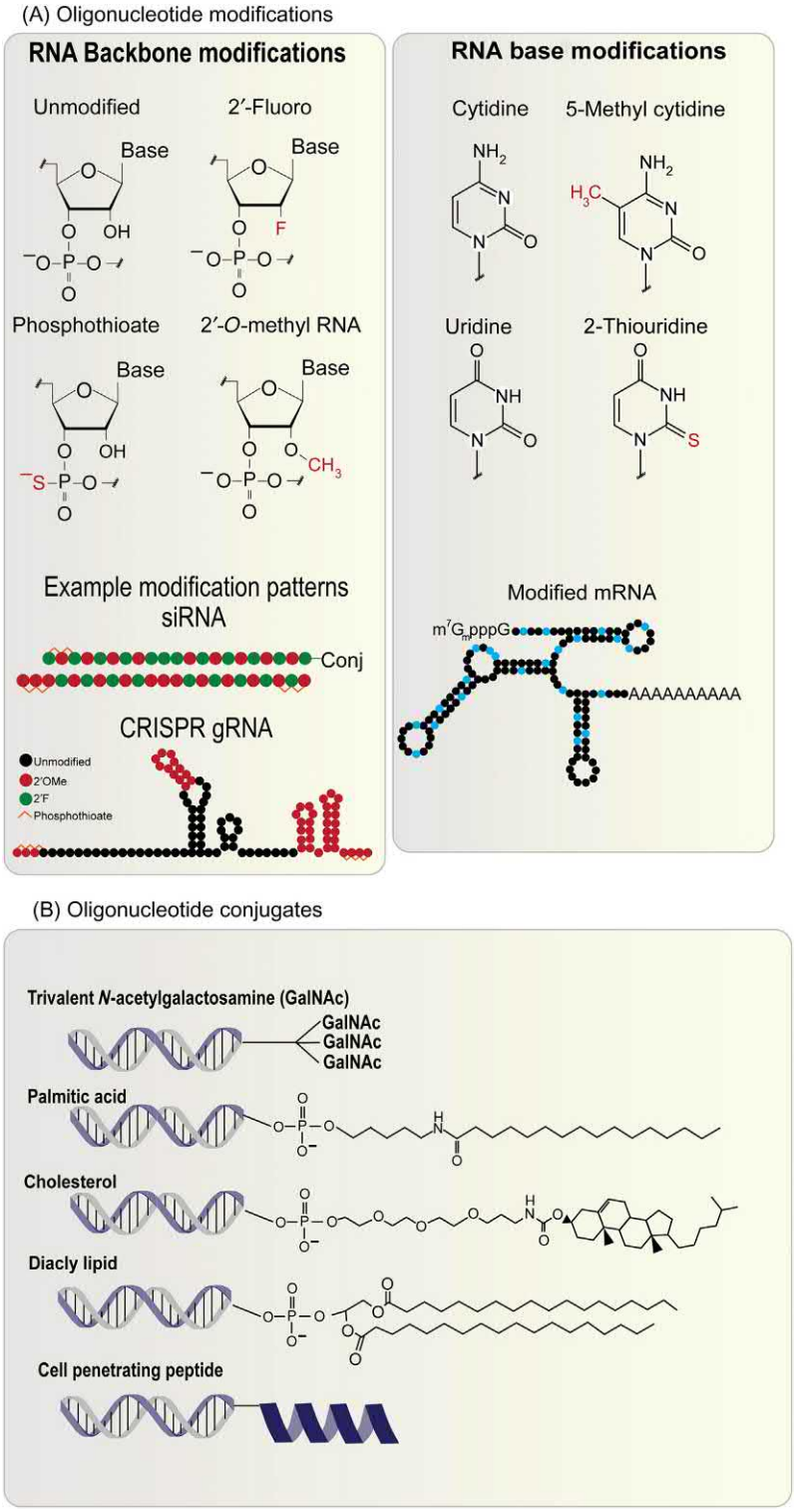

FIGURE 29.6 Modifications used to improve activity and reduce immunogenicity of delivered RNA. (A) Backbone modifications can be used to improve activity, increase nuclease resistance, and reduce innate immunity include 2′-Fluoro, phosphothioate, and 2′-*O*-methyl among others. An example siRNA modification pattern is shown with orange phosphothioate backbone, green 2′F modifications, and red 2′OMe modifications. A highly active modification pattern for CRISPR gRNAs is also shown. Individual bases can be modified to improve mRNA translation and reduce immunity which include 5-methyl cytidine and 2-thiouridine. A schematic of a modified mRNA is shown with ~25% substation. (B) Reported conjugates to siRNA that improve transfection efficiency or pharmacologic properties of IV-administered siRNA include trivalent GalNAc, palmitic acid, cholesterol, diacyl lipid, or cell-penetrating peptides among others. *gRNA*, guide RNA; *siRNA*, small interfering RNA.

from the outermost residues (convergent synthesis). Commonly used, commercial PAMAM forms spherical polymers with good aqueous solubility because of its highly charged, exposed surface groups, which include abundant primary amines for convenient functionalization [166].

The amines can serve three primary functions in these systems: nucleic acid packaging, enhanced cell uptake, and endosome escape. By mixing with polycations in aqueous solutions, DNA and siRNA, with their negatively charged, phosphate-containing backbone, can be

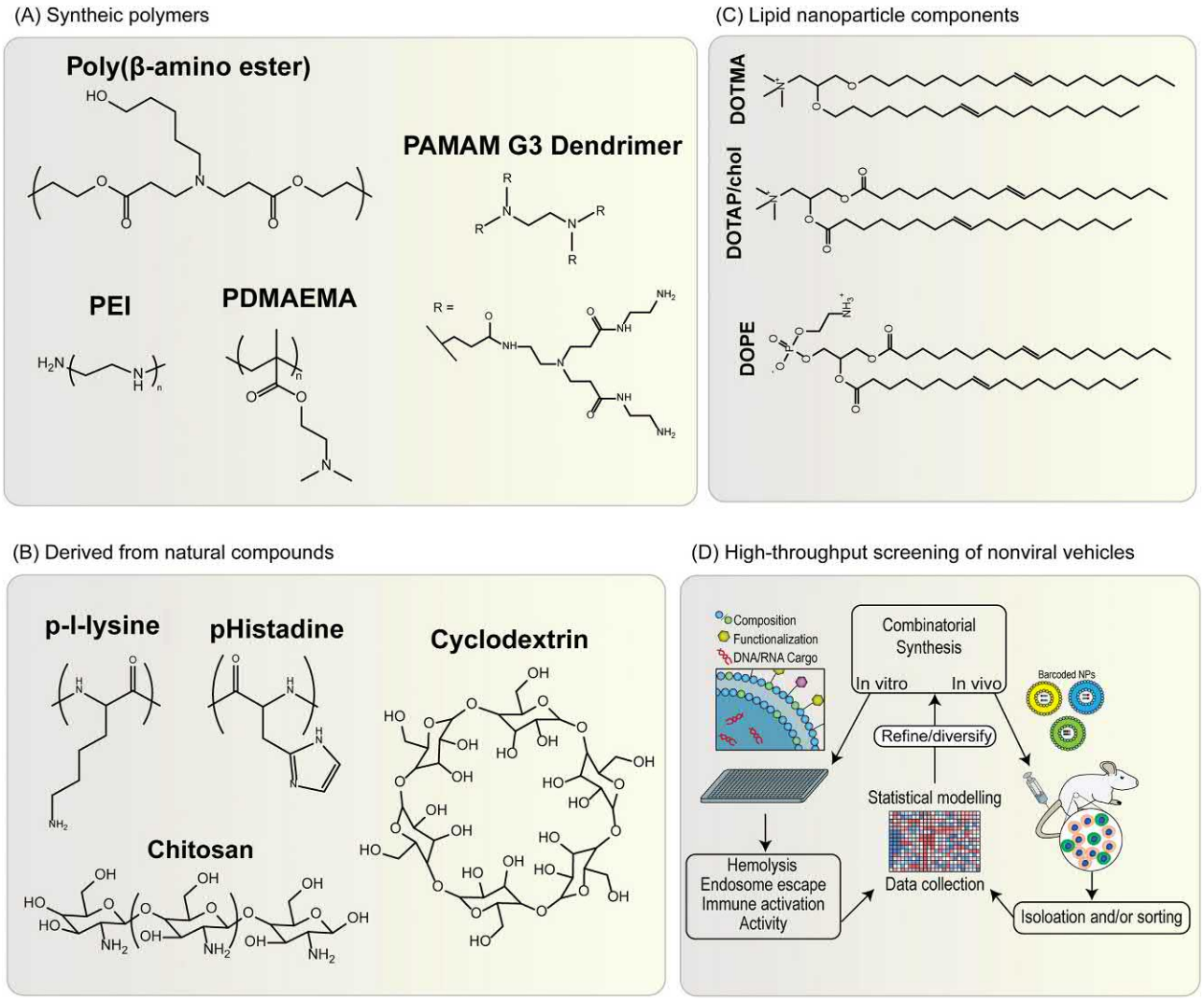

FIGURE 29.7 **Design and optimization of nonviral vehicles for gene delivery.** (A) Several polycationic polymers for nanoparticle production are shown including poly(B-amino esters), PEI, PAMAM dendrimers, and PDMAEMA. (B) Examples of polymers derived from natural components that are used for nucleic acid delivery are shown. These include amino acid-based polymers such as poly(Lys), poly(His), and CPP-containing peptides have been actively tested for delivery applications. Likewise, chitosan and polymers containing β-cylcodextrins and other carbohydrate-containing polymers such as PGAAs have also shown tremendous potential. (C) Lipids traditionally studied for nucleic acid delivery include DOTMA and DOTAP. (D) Recent progress has been made using high-throughput combinatorial approaches for identifying optimal siRNA delivery systems including in vitro assay development or in vivo screening of carriers. Further rounds of optimization can be accomplished by statistical modeling to refine the library or add further library diversification. *CPPs*, Cell-penetrating peptides; *DOTAP*, 1,2-dioleoyl-3-trimethylammonium-propane; *DOTMA*, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; *PAMAM*, poly(amido amine); *PDMAEMA*, poly(2-(dimethylamino)ethyl methacrylate); *PEI*, polyethylenimene; *PGAAs*, poly(glycoamidoamine)s; *siRNA*, small interfering RNA.

electrostatically condensed into particles, termed polyplexes. Typically, an excess of the polycation is used during polyplex formation, yielding particles with an overall net positive charge. The positive surface charge of the polyplexes increases interaction with negatively charged cell membranes, a process that is likely mediated through anionic, heparan sulfate proteoglycans anchored on the cell surface [167]. This binding enhances their endocytotic cell uptake. Following endocytosis, these polyplexes are capable of mediating endosomal escape through the osmotic disruption (e.g., the proposed "proton sponge" effect) [168].

Cationic polymers composed of secondary and tertiary amines, which enable endolysosomal escape through the proton sponge mechanism, can efficiently transfect nucleic acids into cells [169]. Although these net cationic polyplexes can effectively delivery nucleic acids in vitro, they can cause cytotoxicity, and they have a limited biodistribution profile if delivered intravenously. This is because the cationic surface charge of these polyplexes

causes aggregation with serum proteins and red blood cells. These nonspecific interactions can cause disproportionate biodistribution to the capillary beds of the lungs, very short circulation times, and acute toxicity [170]. Thus many strategies have focused on decreasing the cytotoxicity and improving steric stabilization of cationic polyplexes. As discussed above, the incorporation of PEG onto the polyplex surface is an important design aspect for reducing the positive surface charge, improving biodistribution, and decreasing acute toxicity [40,171]. Bioreducible polycation variants that are degraded by the reducing environment in the cell have yielded polyplexes with lower cytotoxicity and higher transfection efficiency [172,173].

Micelles formed from synthetic polymers represent another promising category of nucleic acid carriers [174]. For nucleic acid delivery, the Kataoka group has focused on micelles driven by electrostatic interactions or polyion complex micelles [175]. These nanovehicles typically self-assemble after mixing of nucleic acids with diblock polymers consisting of PEG and a polycationic segment such as poly(L-lysine) [poly(Lys)]. This class of carriers has been shown to achieve gene silencing in vitro, and a variant composed of a diblock polymer of PEG, poly(Lys) and cyclic Arg-Gly-Asp (RGD) targeting peptides efficiently achieved gene silencing and reduced tumor mass following delivery of antiangiogenic siRNA in vivo [176].

Micelles are traditionally defined as core-shell self-assemblies of amphiphilic diblock polymers into morphologies where a more hydrophobic block forms the micelle core, and a more hydrophilic block forms the corona. This class of micelles, which self-assemble into NPs in aqueous solutions even in the absence of added nucleic acid, has shown great promise [177–179]. One example of a micelle-forming polymer is composed of a block of PDMAEMA and a second block that consists of a random terpolymer of 50% butyl methacrylate (BMA), 25% DMAEMA, and 25% propylacrylic acid (PAA). This polymer self-assembles into micellar NPs of approximately 50 nm in diameter, with the poly(BMA-*co*-DMAEMA-*co*-PAA) terpolymer block in the particle core. The 50 mol.% of the hydrophobic BMA drives self-assembly, and electrostatic interactions between PAA and DMAEMA also help to stabilize the micelles near physiologic pH. The homopolymer block of DMAEMA forms the micelle corona and provides a cationic surface that can be used to electrostatically condense siRNA into serum stable siRNA-NPs, sometimes referred to as micelleplexes. DMAEMA and PAA monomers both contain protonatable groups (tertiary amine and carboxylic acid, respectively) with pK values approximately equal to physiologic pH. The net charge of the anionic PAA and cationic DMAEMA is relatively balanced at physiologic pH, stabilizing the micelle core. However, due to concurrent protonation of DMAEMA and PAA, the core-forming terpolymer block acquires a net positive charge when it enters the acidic endolysosomal pathway. This causes a shift to a net cationic state that electrostatically destabilizes the micelle core, exposing the terpolymer block, and activating its membrane disruptive activity. This terpolymer composition has been fine-tuned for intracellular delivery based on the BMA content [178] so that this pH-driven transition occurs in environments representative of the early and late endosomal compartments.

The micelles described above have a cationic surface charge that is not amenable to effective intravenous delivery because, as described for PEI and PDMAEMA homopolymers, particles with cationic surfaces have short circulation times. As discussed above, reversible PEGylation can be utilized to create more stealthy cationic carriers in the circulation while maintaining key, underlying functionalities. For example, a variant of this micelle has been created with a PEG corona that can be removed by naturally occurring proteases for "proximity-activated targeting" [45,180–183]. The intact PEG corona reduces nonspecific binding and uptake in the circulation, while high MMP activity at the pathological site releases the corona and triggers activation of the underlying siRNA-NP.

Evidence suggesting that toxicity profiles are improved with biodegradable versions of traditional polycations has spurred the development of new and better optimized biodegradable polymer chemistries. PBAEs are one very promising class of biodegradable polycationic nucleic acid carriers that have been shown to be superior to in vitro transfection reagents such as Lipofectamine 2000 [184]. A key characteristic of PBAEs is that they are amenable to parallel, high-throughput synthesis for simultaneous screening of large numbers of polymer variants. Using this approach, PBAE compositions have been identified with transfection activity superior to other nonviral agents and that rival the performance of viral vectors in some applications [185,186]. Conveniently, PBAE-based carriers rapidly hydrolyze and degrade into low molecular weight diols and bis(β-amino acids) in response to the pH drop that occurs during endosomal/lysosomal trafficking, which both facilitates nucleic acid release and makes them less cytotoxic than polymers like PEI [187].

Polymers derived from natural sources or monomers

Amino acids and saccharide-based materials have been extensively explored for nucleic acid delivery, as more thoroughly reviewed elsewhere [188,189]. These natural

building blocks have the potential to have reduced cytotoxicity and, if optimized, superior overall function relative to fully synthetic biomaterials. Here, peptide-based and carbohydrate-based nucleic acid delivery polymers will be surveyed (Fig. 29.7B).

The first amino acid–based polymer to be pursued was poly(Lys) [190], and many iterations of this cationic polymer have been extensively studied in a fashion similar to PEI and the other synthetic polycations described above. Poly(Lys) can efficiently complex nucleic acids, but its transfection efficiency is low, and it requires cell treatment with endosomolytic agents such as chloroquine to enhance gene expression. Nonetheless, the early studies on poly(Lys) yielded important mechanistic insights related to polyplex formulation, intracellular trafficking, and endosome escape [190]. While the clinical promise of poly(Lys) is limited, these early studies have had a significant impact on the field.

Poly(histidine) (poly(His)) is another amino acid–based polymer that has shown some usefulness for gene therapy. The amino acid His has an imidazole R-group containing a secondary amine that endows poly(His) with proton sponge activity for endosomal escape [191]. Polymers containing both Lys and His have also been utilized successfully in combinations. In these hybrid polymers, the primary amines on Lys are fully protonated and cationic at physiologic pH, enabling efficient electrostatic complexation with DNA. The lower pKb of the secondary amines from His provide complementary proton sponge activity for endosome escape [192]. The transfection efficiency of polymers with poly(His) grafted to poly(Lys) is significantly improved by the addition of the endosomal disruption agent chloroquine, indicating that the polymers alone are still partially prone to endosomal entrapment [193]. Highly branched architectures of His/Lys polymers and His-containing reducible polycations have also been found to efficiently deliver siRNA [194,195]. There is also a precedent for incorporation of amino acid–based subunits into other gene therapy systems in order to produce "hybrids" with enhanced delivery functionality. For example, His has also been used to modify chitosan (discussed more next) to enhance its endosomal escape and transfection efficiency [196].

Cell-penetrating peptides (CPPs) and pH-responsive, fusogenic peptides are two other classes of peptides that have been rigorously explored to trigger cell uptake and endosomal escape, respectively. These peptide classes have been used both in combination and as components of multifunctional polymer and liposomal delivery systems. Most CPP and fusogenic peptides are derived from bacterial toxins and viral vectors, or they are synthetic analogs of the naturally occurring peptides. The *trans*-activating transcriptional factor of HIV-1 [197], and the antennapedia peptide derived from *Drosophila* [198] are two examples of well-studied CPPs. These peptides are typically rich in cationic amino acids, and as a result, synthetic, arginine-rich CPPs of various types have also been found to mediate biomacromolecular cargo cell uptake. Fusion of a CPP with a dsRNA-binding domain has been used to deliver siRNA into primary cells considered difficult to transfect [199]. Other CPPs derived from Transportan 10 (designated PepFect) [200] and CADY [201] have been designed for efficient intracellular delivery. Fusions of tumor-penetrating and CPPs have been generated that have shown silencing of an oncogene improved survival in mice [202,203] The ability of CPPs to trigger cell internalization has been leveraged for delivery of several classes of therapeutic cargo including plasmid DNA and siRNA (see CPP reviews for additional information).

Fusogenic peptides are pH-responsive peptides that can fuse with or form pores through the endosomal membrane. An example is the diphtheria toxin, which has a subunit that forms transmembrane pores in endosomes that enable entry of a disulfide-linked toxin fragment into the cytosol [204]. Another example is hemagglutinin, an influenza protein that creates pH-dependent endosomal membrane fusion to deliver the viral genetic material into the cytoplasm [205]. The peptide GALA is a synthetic, pH-dependent, fusogenic peptide that has been extensively characterized [206]. GALA self-assembles and inserts into lipid bilayers at acidic pH, forming a pore that allows of the membrane transit [207]. For example, GALA has been successfully applied to enhance efficiency of cytosolic delivery of nucleic acid cargo packaged in PAMAM and liposomes [208–210].

The polysaccharide chitosan, oligosaccharides-like cyclodextrins, and a variety of other saccharide-containing glycopolymers represent another polymer class for nucleic acid delivery. For example, natural anionic saccharide–based polymers can be fabricated into thermodynamically stable, polyelectrolyte complex (PEC) NPs through spontaneous association triggered via mixing of polyelectrolytes of opposite charge, as reported by Prokop et al. [211,212]. Typically, PEC NPs are made by mixing polyanionic core polymers, such as alginate or chondroitin sulfate with corona polycations such as spermine hydrochloride or poly(methylene-*co*-guanidine) hydrochloride. This multipolymeric nanoparticulate approach has been shown to be effective for gene transfer in vitro [213], particularly in cell systems that are normally refractory to gene transfer, such as pancreatic islets and antigen-presenting cells. In addition, PEC coronal surfaces can be decorated with PEG-ligand complexes to increase cell targeting and reduce nonspecific uptake.

Chitosan, a polysaccharide composed of glucosamine and N-acetyl glucosamine units bonded via $\beta(1\rightarrow4)$ glycosidic bonds, is one of the most thoroughly studied saccharide polymers. Chitosan benefits from being a "green"

approach, because it is a renewable resource derived from chitin. This natural polymer is also biodegradable and nontoxic. The Alonso laboratory introduced chitosan-based NPs [214] made via ionotropic gelation, based on the interaction between the negative groups of pentasodium tripolyphosphate and the positively charged amino groups on chitosan. The chemistry of chitosan is also adaptable to nonviral gene therapy, since it contains several primary and secondary amines capable of endosomolysis via the proton sponge effect. Therefore chitosan has been examined as a pH-responsive polymer for nucleic acid delivery. Howard et al. employed chitosan NPs containing siRNA to knock down enhanced green fluorescent protein (eGFP) in both H1299 human lung carcinoma cells and murine peritoneal macrophages (77.9% and 89.3% reduction in eGFP fluorescence, respectively) [215]. The chitosan NP has a high potential for transmucosal delivery. Effective in vivo RNAi was achieved in bronchiolar epithelial cells of transgenic eGFP mice after nasal administration of chitosan/siRNA formulations (37% and 43% reduction as compared to mismatch and untreated control, respectively). The principal drawbacks of chitosan are poor solubility in physiological buffers and lower endosomolytic activity compared to some stronger proton sponge polymers. As a result, several variants of chitosan have been made with modifications to increase endosomal escape and solubility. For example, PEI and imidazoles have both been conjugated to chitosan to enhance its performance in gene therapies [216].

Cationic polymers containing beta-cyclodextrins (β-CD) showed early promise for clinical RNAi. Cationic β-CD-based polymers (βCDPs) synthesized by the condensation of a diamino-cyclodextrin monomer with a diimidate comonomer are capable of forming polyplexes with nucleic acids, and their transfection performance depends on βCDP structure [217]. The β-CD-containing polycations are especially unique because cyclodextrins contain an interior cavity that can be used to form inclusion complexes with hydrophobic moieties. For example, β-CD binds tightly to the hydrophobic molecule adamantine, and this provides a convenient "handle" from which to functionalize the surface polyplexes made from βCDPs with PEG or targeting ligands [218,219]. The Davis laboratory translated this concept from benchtop to clinical trials [220]. This carrier was the basis for a report demonstrating the first example of human RNAi using targeted polymeric NPs. This carrier was composed of βCDPs functionalized with both PEG and the cancer-targeting ligand transferrin [221]. After this landmark finding the clinical trial was ended primarily owing to dose-limiting toxic events [222] with no follow-on trials. Arrowhead Pharmaceuticals subsequently focused on conjugates and no follow-on phase III clinical trials were initiated.

A variety of other novel, synthetic cationic glycopolymers are also in the developmental pipeline for clinical applications of nucleic acid delivery [189,223]. The Reineke lab has made key contributions in this area (see recent review Ref. [224]), and an example class of glycopolycations developed by this group are the poly(glycoamidoamine)s (PGAAs) [223]. A library of PGAAs was made through the condensation reaction between carbohydrate and oligoamine comonomers. These PGAAs were varied based on a variety of parameters, including the carbohydrate size, the hydroxyl number and stereochemistry, the amine number, and whether or not heterocyclic groups were present. These polymers have been screened for gene delivery, and optimized formulations have been identified that facilitate efficient DNA packaging and intracellular delivery properties. The Reineke group has also sought a variety of trehalose-based polymers, and promising results continue to suggest the potential for clinical translatability of this safe and efficient class of polymers [225]. This glycopolymer has also been adapted to deliver CRISPR-based transcriptional activator [226].

Exosomes are cell-derived extracellular vesicles contain a variety of nucleic acid types. These natural vehicles participate in cell communication and are increasingly being used in drug and gene delivery [227]. A major challenge in utilizing exosomes for gene delivery is loading of the vehicles with nucleic acids. While electroporation is the more common method of loading DNA, sonication, extrusion, and freeze-thaw cycles are other methods for loading exosomes [228]. Loading of exogenous siRNA or DNA remains a challenge owing to low efficiency and aggregation during electroporation. For regenerative medicine, exosomes derived from stem or progenitor cells may encourage tissue regeneration on their own without exogenously delivered DNA including muscle regeneration [229], wound healing [230], angiogenesis, and cartilage repair [231].

Lipid-based delivery systems

Lipids are one of the most commonly used approaches for nucleic acid transfection. Lipid agents can form small, artificial, spherical liposomal vesicles with a lipid bilayer membrane surrounding an aqueous interior. Liposomes can be produced from natural nontoxic phospholipids and cholesterol. Liposome properties vary substantially with lipid composition, size, surface charge, and the method of preparation. Because of their size, hydrophobic and hydrophilic compartments, as well as biocompatibility, liposomes are promising systems for drug delivery. Hydrophilic drugs can be encapsulated, or cationic lipids can be used to form lipoplexes with anionic nucleic acids. Like other nucleic delivery systems discussed,

PEGylation can be used to shield liposomal surfaces to reduce nonspecific protein and cell interactions and to improve circulation time [232]. Targeting of liposomes has also been accomplished by anchoring a variety of targeting ligands, such as antibodies, to the liposomal surface [233]. The earliest approaches to lipid-based nucleic acid delivery focused on cationic lipids such as *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (Fig. 29.7C) [234].

A large number of IV-delivered siRNA drugs that have advanced into clinical testing to date employ lipid-based delivery systems, with the LNP delivery technology providing the first clinically approved product, patisiran for siRNA delivery. Langer and Anderson have been significant contributors to the literature on optimization of lipid-like materials for delivery of siRNA through a series of high-throughput combinatorial approaches seeking to identify optimized lipid-like materials, or lipidoids, for siRNA delivery. In some of the first published work using this approach, they synthesized nearly 700 lipidoids based on the conjugate addition of alkyl-acrylates or alkyl-acrylamides to primary or secondary amines [235]. This library featured compounds with varied alkyl chain length, linker degradability, amine R-groups, and postsynthesis quaternization of the amine to induce stable cationic charge. The resulting lipidoids were used to package siRNA and screened for optimal transfection efficiency in vitro and in vivo. In these studies, efficient gene silencing activity was shown in both rodents and nonhuman primates at doses of 2.5 mg/kg and greater. In a follow-up paper, they synthesized a library of 126 lipid-like compounds using a unique synthetic strategy based on epoxide chemistry [236]. Their screen in this study identified some of the most potent RNAi delivery compounds produced to date, and their best lipidoids produced liver-specific silencing at 0.01 mg/kg in mice and at 0.03 mg/kg in nonhuman primates. Similar work has also been utilized recently to identify lipidoids for transfection of pDNA, and compounds were identified that were superior to Lipofectamine 2000, which is a gold standard of commercially available in vitro transfection reagents [237]. Delivery of CRISPR/Cas9 mRNA has been accomplished with LNPs in vivo with the observation that the short duration of exposure of CRISPR/Cas9 components maximizes the ratio of on to off target ratio editing relative to sustained expression [238]. One of the limitations to much of the lipidoid work is that it has been primarily limited to liver targets; however, high-throughput screens are beginning to reveal other tissue targets [239]. The promising current data suggest that clinical success will be established first in the liver and that subsequent work will optimize this class of carriers for additional clinical applications.

Inorganic nanoparticles

In some applications, inorganic NPs, "theranostic" technological advances have been pursued that combine both image contrast and therapeutic functionalities. For example, quantum dot NPs, recognized for their optical properties, have been functionalized to successfully deliver siRNA [240,241]. NPs that provide MRI contrast have also been successfully utilized to simultaneously provide image contrast and siRNA delivery [242]. Recently, gold NPs have come to the forefront of inorganic NP approaches for siRNA delivery. Mirkin has been a leader in this field through development of polyvalent siRNA conjugates on the surface of gold NPs [243]. These particles are efficiently internalized by pattern-recognition scavenger receptors and can mediate gene silencing without addition of any additional transfection reagents [244]. Interestingly, these gold NPs surrounded by a dense shell of covalently bound siRNA have been recently found to efficiently penetrate the epidermal layer of the skin, indicating that they may be especially useful for clinical applications where topical delivery is a logical approach [243].

These skin-penetrating NPs may obviate the need for the previously popular biolistic particle delivery systems. This older method used a handheld gene gun with a pulse of helium to fire gold particles coated with DNA into target cells. This method of transfection is an effective physical means of rapid plasmid delivery into mammalian tissue. It is generally restricted to local expression in the dermis, muscle, or mucosal tissue since the gold particles are shot into confined tissue sites. Transfection depth is limited to about 1 mm, and about 10% of the cells in the tissue (skin) can be transfected. From a translational perspective, the DNA-loaded particles have a relatively long shelf life, and they have been successfully used for cutaneous administration of growth factor/receptor constructs [245–247].

Inorganic NPs may be better suited for delivery of uncharged cargo including CRISPR ribonucleoproteins (RNPs) or PNAs. As described above, porous silicon NPs have been adapted to deliver PNAs to hepatocytes in vivo based on the large capacity and ability to load the uncharged PNAs [156]. In addition, two papers described the utility of gold NPs for delivery of CRISPR RGNs and DNA repair template in a mouse model of DMD and fragile-X syndrome [248,249].

High-throughput screening

High-throughput screening has been applied to find more active nonviral gene carriers with improved pharmacological properties (Fig. 29.7D). High-throughput in vitro screening takes advantage of advances of combinatorial

parallel synthesis of carrier precursors and high-throughput screening readouts to examine the efficiency of thousands of carriers [235,250,251]. Other assays can be used to screen large libraries in vitro for safety and efficiency before animal experiments including hemolysis [252], stability in serum [253], and galectin 8 intracellular tracking [254]. High-throughput screening can be combined with machine learning to optimize multiple design parameters at once as recently demonstrated by the Mirkin group with the spherical nucleic acid delivery platform [255].

Another approach uses barcoded DNA encapsulated within libraries of nonviral carriers that can be screened directly in vivo for efficient delivery or activity in the tissue of interest [239]. This approach has the potential to find nonviral carriers with unique target tissues and can dramatically expand the number of particles with in vivo efficiency data. A combination of approaches can be used to find carriers that are safe for systemic administration and efficient for gene delivery.

High-throughput genetic screens have been enabled by CRISPR-Cas technologies. Genome-wide libraries are available to screen cells based on a custom reporter output, proliferation, cell death, or other readout [256–258]. CRISPR gRNAs can be paneled on genes of interest to examine functional genetic elements or nuclease deactivated CRISPR can be fused with transcriptional activators or repressors to screen for effects of gene silencing or gene activation [259–261]. Paired with advances in single-cell sequencing [262], these technologies can reveal mechanistic insights in tissue regeneration. The resulting information from these screens can reveal new targets for gene therapy or targets for small molecule drug development [12].

Engineering tissues with gene delivery

Introduction to engineering tissue with gene delivery

Multiple gene delivery approaches have been used to engineer tissues, including ex vivo and in vivo genetic manipulation of cells. The most developed applications include oligonucleotides for muscle gene therapy, gene therapy to improve cardiac regeneration, regenerating skin, bone, and others. In contrast to systemic applications where circulation time and target cell uptake is maximized, tissue engineering approaches typically bypass systemic barriers and depend on rapid cell recognition and intracellular delivery.

Viral delivery to engineer tissues

The most common approach to viral gene delivery for tissue engineering is to genetically modify the cells in standard cell culture, and then subsequently seed them onto a three-dimensional scaffold [263,264]. However, this approach involves extra procedures and in vitro manipulation of the cells that adds significant complexity to an expensive and work-intensive process. Therefore there has been considerable effort in functionalizing biomaterial scaffolds with viral vectors, such that cell seeding onto the scaffold and cell transduction by gene delivery vehicles becomes a single procedure [265]. This approach may also enhance transduction efficiency by colocalizing the cells and virus and also protect them from the immune system. Patterns or gradients of viral vectors can be generated to spatially control gene transfer and tissue development. Material-mediated gene delivery also preserves and potentially isolates the vector from the immune system and controls the localization of the virus. These approaches typically involve either encapsulation of the virus within a hydrogel, immobilization of the virus to a surface, or a combination of these strategies. The unique biological properties of each virus require distinct techniques for each vector.

The incorporation of adenovirus into hydrogels or protein matrices has led to control over the spatial and temporal delivery to surrounding tissue while also protecting the virus from loss of activity [266,267]. Delivery of therapeutic genes from hydrogels can also enhance the wound healing properties of materials such as fibrin [268] and collagen [269]. This approach has been particularly successful in promoting the healing and integration of dental implants in animal models, suggesting a promising application in oral defects and disease [270]. Adenovirus may also be bound to exposed surfaces of polymer scaffolds via conjugation of antiadenovirus antibodies to chemically reactive surfaces [271] or the use of biotinylated adenovirus [272]. This strategy can be used to spatially organize gene transfer by controlling chemical functionalization [273]. Adenovirus has also been widely used in gene-eluting stents and heart valves to prevent restenosis and valve disease [274–276].

Schwarz et al. have shown that the enhanced stability of AAV vectors can be exploited to create biomaterials, allografts, and other tissue substitutes with freeze-dried AAV coatings that are taken up by cells after implantation. The coating of bone allografts with osteogenic and angiogenic factors encoded by freeze-dried AAV vectors led to increased bone healing [277]. The same approach has also been used to engineer bone tissues in vitro by coating allografts [278] or polymer scaffolds [279].

The electrostatic properties of the retroviral and lentiviral lipid bilayer can modulate virus interactions with biomaterials, leading to enhanced and controlled transduction of cells [280]. This approach has been used to create gradients of retroviral particles on three-dimensional scaffolds that encode genes that direct tissue formation,

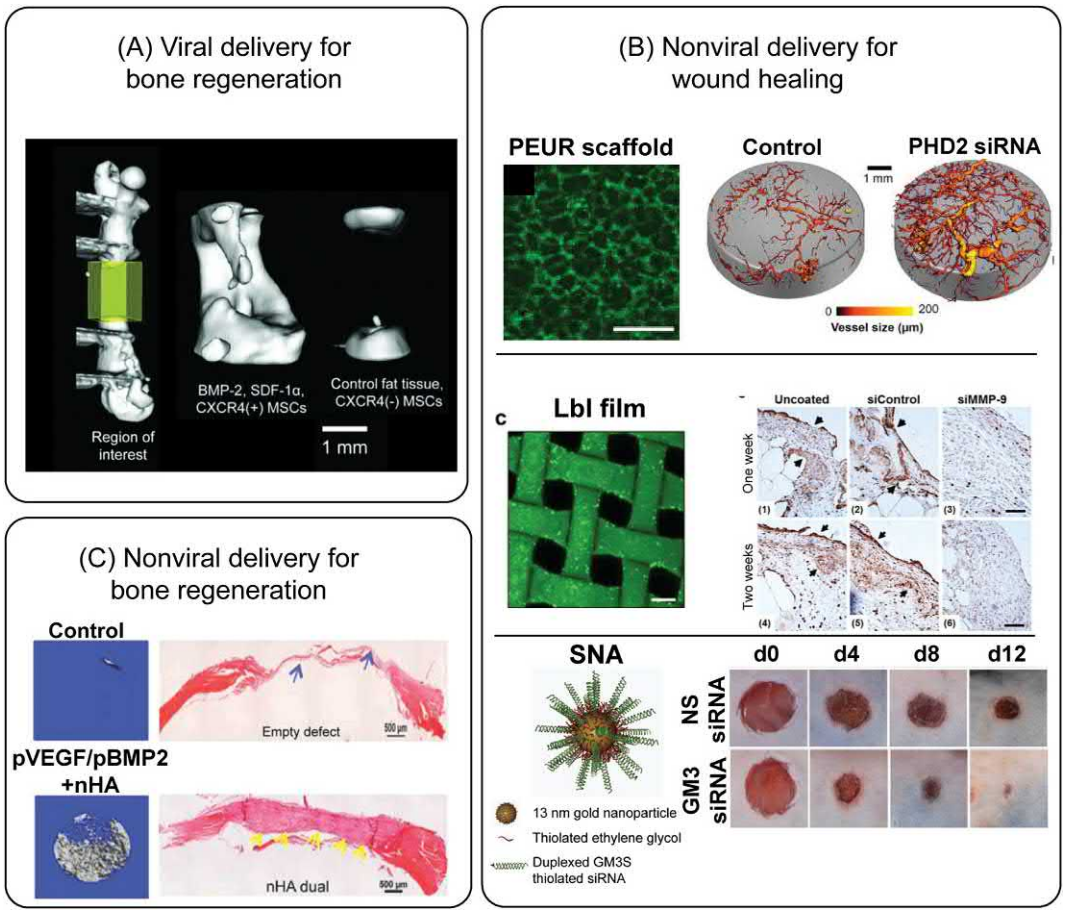

FIGURE 29.8 **Promising preclinical data for tissue engineering includes viral and nonviral delivery.** (A) MSCs transduced to overexpress CXCR4 were implanted and Adenovirus delivering BMP-2 and SDF-1α was delivered showing improvements in bone formation in a critical defect model. (B) Several approaches to heal skin wounds with nonviral delivery of siRNA have been developed. The first example used polymeric nanoparticles loaded into a PEUR scaffold with siRNA targeting PHD2 to increase angiogenesis. MicroCT shows increase angiogenesis relative to the control group. The second example used layer-by-layer deposition of siRNA to decrease MMP-9 expression and improve wound healing in a diabetic mouse model. The last example shows the use of spherical nucleic acids to silence GM3 to improve wound healing in a diabetic mouse model showing more rapid wound closure by 12 days. (C) Nonviral plasmid DNA delivery was used to regenerate bone in a mouse defect model. MicroCT images show restored bone growth when both VEGF and BMP2 plasmids are delivered with nHA particles from a collagen scaffold. *siRNA*, Small interfering RNA. *Reprinted with permission from Curtin CM, et al. Combinatorial gene therapy accelerates bone regeneration: non-viral dual delivery of VEGF and BMP2 in a collagen-nanohydroxyapatite scaffold. Adv Healthc Mater 2015;4,223–7, doi:10.1002/adhm.201400397; Nelson CE, et al. Tunable delivery of siRNA from a biodegradable scaffold to promote angiogenesis in vivo. Adv Mater 2014;26:607–14, 506, doi:10.1002/adma.201303520; Castleberry SA, et al. Self-assembled wound dressings silence MMP-9 and improve diabetic wound healing in vivo. Adv Mater 2016;28:1809–17, doi:10.1002/adma.201503565; Randeria PS, et al. siRNA-based spherical nucleic acids reverse impaired wound healing in diabetic mice by ganglioside GM3 synthase knockdown. Proc Natl Acad Sci USA 2015;112:5573–8, doi:10.1073/pnas.1505951112.*

providing a means for more complex tissue formation that better mimics natural development [281]. Ionic interactions can also be used to load lentivirus onto hydroxyapatite NPs that protect the virus to enable incorporation into hydrogels [282,283]. Lentivirus has also been immobilized onto a variety of other materials with various properties that can be tailored for controlled gene transfer in vitro and in vivo [284,285]. Viral vectors can also be used to directly transduce cells in local pathologies including articular cartilage, muscle, bone, and regenerating skin [286–288]. For example, a combinatorial therapy of mesenchymal stem cells (MSCs) expressing CXCR4 and adenoviral delivery of BMP-2 and SDF-1α was able to significantly improve bone regeneration in a mouse critical defect model (Fig. 29.8A) [289]. A recent study from the Belmonte lab used AAV as a gene delivery vector to reprogram cells in vivo to improve wound healing. In this study, the authors delivered four transcription factors to reprogram mesenchymal cells into a more pluripotent state which assisted epithelializing the wounds in animal models. Four transcription factors (DNP63A, GRHL2, TFAP2A, and MYC) delivered by AAV serotype DJ were used to reprogram cells to improve wound closure [290].

Nonviral delivery from scaffolds

Local, nonviral delivery of nucleic acids from biomaterial scaffolds presents a promising approach for tissue regeneration. Scaffold-based delivery can provide an efficient means to stimulate local, site-specific effects without the need for targeting and other challenges presented by systemic, intravenous delivery. These approaches can be designed for both delivery of gene-encoding plasmids and RNAi gene silencing through delivery of siRNA or plasmids that encode shRNA. These local, nonviral gene therapies have applicability for improving repair of a variety of tissues such as skin wounds, bone defects, and myocardial infarcts.

There is a relatively established precedent for scaffold-mediated plasmid delivery for increasing expression of growth factors such as platelet-derived growth factor (PDGF) to induce blood vessel and tissue formation within the scaffold [291]. It has been shown that embedding plasmid DNA within a biodegradable tissue scaffold or immobilizing it on a biomaterial surface can provide a 10−100-fold increase in transfection efficiency because of increased local concentration and extended retention of plasmid at the cell-biomaterial interface relative to a local injection of plasmid-containing solution [291−293]. Mechanistically, "substrate-mediated delivery" of nucleic acids is believed to operate through multiple modes of endocytosis, though caveolae-mediated uptake may play the largest role [294]. This bioinspired approach to tissue regeneration is also analogous to the pathways that viruses "hijack" when they attach to extracellular matrix as a strategy to increase their cell internalization and infection [295]. The seminal work on scaffold-based plasmid delivery by Mooney and Shea has been subsequently applied in a diversity of applications, including delivery from both natural [296] and synthetic biomaterials [297,298]. Through more complex approaches to surface immobilization, plasmid delivery systems can also be engineered to control spatial patterning, concentration gradients, and temporal profiles in 2D and 3D [299]. Work is also underway to optimize the therapeutic benefits of substrate-mediated delivery by using enzymatically labile tethers [300], cell-specific targeting proteins [301], and other means of cell-specific targeting and intracellular release.

Scaffold-based local RNAi through delivery of siRNA is a more recent development and was initially approached primarily using natural biomaterials such as alginate, collagen, and agarose [302−304]. The first in vivo studies demonstrated gene silencing in skin wounds using Lipofectamine 2000 siRNA lipoplexes embedded in agarose gels. These commercially available cationic lipoplexes were released in a rapid burst and were effective for short-lived, topical siRNA application [304,305]. Prefabricated, electrospun scaffolds made from ε-caprolactone and ethyl ethylene phosphate copolymer nanofibers have also been pursued for the release of siRNA/transfection reagent complexes and have been shown to achieve more sustained delivery of bioactive siRNA [306].

The Duvall lab adapted a polyester urethane PE-UR scaffold-based platform for local delivery of siRNA-NPs in regenerative applications [307,308]. The PE-URs can be fabricated using a two-component foaming process that allows injection into a defect, followed by rapid curing in situ to provide mechanical support for tissue in-growth [309] and biodegrade hydrolytically and oxidatively into biocompatible side products at tunable rates [310]. By delivering siRNA targeting PHD2, angiogenesis was significantly increased in a mouse model with release rates tunable by the scaffold formulation (Fig. 29.8B) [308]. The scaffold can be formulated with a reactive oxygen species−degradable linker that provides on-demand degradation and improved tissue granulation leading to improved wound healing in a rat model of wound healing [311].

Another example from the Hammond lab used layer-by-layer scaffolds to locally silence either MMP-9 to improve wound healing [312] or connective tissue growth factor (CTGF) to reduce scarring [313]. The layer-by-layer platform allows the functionalization of commercially available wound dressings with chitosan-siRNA NPs with release profiles that are tunable based on the number of layers leading to improve wound closure (Fig. 29.8C) [312]. To improve scarring in a burn wound model, commercially available silk sutures can be subjected to the same layer-by-layer process loading CTGF siRNA with chitosan-reducing fibrosis and scarring [313]. miRNA inhibition can be used to improve wound healing. One study delivered a LNA targeting miRNA210 with lipid NPs injected intradermally to improve ischemic wound closure [314]. Another example for wound healing used siRNA targeting ganglioside-monosialic acid 3 synthase delivered by gold NPs suspended in in the commercial ointment Aquaphor, which improved wound healing in a diabetic mouse model (Fig. 29.6B) [315].

For bone regeneration, gene delivery can be used to improve osteogenesis either by overexpression of target genes or siRNA to silence target genes in stem cells encapsulated in a biomaterial. For overexpression, BMP-2 and VEGF plasmid DNA were encapsulated within collagen-nanohydroxyapatite scaffolds and implanted in vivo showing complete bridging of a bone defect model in rats 4 weeks after administration (Fig. 29.8D) [316]. Another approach used siRNA targeting Noggin or miRNA-20a embedded within PEG hydrogels which improved osteogenic potential of MSCs encapsulated in the hydrogel [317].

Nucleic acid delivery for tissue engineering advances into the clinic

Clinical development of genetic medicine for tissue engineering is ongoing in numerous preclinical studies. Significant progress in the last decade, specifically in cancer therapy and inherited disorders, indicates a groundswell of gene therapy products in the pipeline. Currently approved products include viral vectors, antisense oligonucleotides, cell therapy products, and a LNP-delivered RNAi product. Alipogene tiparvovec (Glybera), the first approved gene therapy product approved in Europe, used an AAV for LPLD, however was withdrawn in October 2017 owing to the high cost and low usage rate. Strimvelis is a gamma-retroviral vehicle for ex vivo stem-cell therapy to replace the defective gene for ADA-SCID. Luxturna is an AAV gene therapy to replace RPE65 to treat Leber Congenital Amaurosis-type 2 and Onasemnogene abeparvovec is an AAV gene therapy that was recently approved for spinal muscular atrophy. In 2016 two splice-modulating oligonucleotides delivered without a carrier were approved. Exondys 51 (eteplirsen) is a carrier-free oligonucleotide injection that restores marginal dystrophin protein expression in Duchenne muscular dystrophy in patients amendable to exon 51 removal (~13% of DMD patients) [8]. Shortly after, nusinersen was approved for spinal muscular atrophy, which is delivered by intrathecal injection which increases the local concentration and extends the half-life from days to months [9]. In 2018 OnPattro (patisiran) was the first RNAi drug approved. Onpattro is an siRNA delivered by lipid complex to treat hereditary ATTR amyloidosis by silencing abnormal transthyretin [2] with other RNAi approaches advancing through clinical trials targeting PCSK9, antithrombin, and delta aminolevulinic acid synthase 1 [318–320]. The accelerating pace of gene therapy clinical work and approved products signals that the gene therapy field is coming to maturity and on the cusp of providing significant clinical impact.

Despite the promises of gene therapy, there currently is no approved gene delivery product for improving tissue regeneration. Although a promising approach, there have been numerous approaches for gene therapy in wound regeneration by delivering gene-encoded growth factors via multiple vectors but have not yet translated to an approved product [321]. Early results showed that adenovirus that expresses rhPDGF-BB to improve bone and skin regeneration [269] was shown to be equally effective as the scaffold alone when compared to standard of care [322]. Similarly, cardiac regeneration has been pursued through intramyocardial delivery of plasmid or viral vectors expressing growth factors but previous clinical trials have suffered from inefficient delivery despite a good safety profile [323].

Gene therapy for rheumatoid arthritis and osteoarthrosis has advanced into clinical trials with one approved product in Korea (Invossa) which is under close scrutiny as of this writing owing to irregularities in the cell preparation. Other approaches in the clinical pipeline include AAV delivery of Etanercept and IFN-β for rheumatoid arthritis and IL-1Ra for osteoarthristis [324]. An IND-enabling study indicated that a self-complementary AAV2.5 expressing IL-1Ra was safe and effective in a rat model of osteoarthritis [325]; this technology is expected to begin phase I clinical trials in 2019 (NCT02790723).

Moderna recently published the results of a phase 1/2 trial using modified VEGF mRNA to treat vascular deficiencies in men with type 2 diabetes mellitius. Intradermal injections of modified mRNA carrier-free led to nearly double VEGF-A protein levels at the higher dose of 360 μg and a statistically significant blood flow increases at day 7 and 14 measured by laser doppler fluximetry (Fig. 29.9A) [153]. A previous preclinical study showed the eventual application of VEGF-A mRNA delivery may be broad including cardiovascular regeneration, diabetic wound healing, and others [152].

One of the most remarkable clinical results was the gene therapy treatment of junctional epidermolysis bullosa with autologous keratinocytes transduced by a retrovirus encoding laminin-332. The study enrolled a single 7-year-old patient with complete epidermal loss on 80% of his body. After three autologous transplantations delivered by plastic- of fibrin-cultured grafts resulted in near complete epidermal regeneration that remained intact through the 21-month follow-up. Biopsies taken at follow-up visits indicated restoration of laminin-332 expression in the patient epidermis-dermis junction (Fig. 29.9B) [326]. This study also demonstrated the feasibility of repopulating the entire epidermis with genetically modified stem cells. Various forms of epidermolysis bullosa affects nearly 500,000 people worldwide, and this approach could be applied to many of them with several clinical trials underway (NCT03490331, NCT02984085).

Future challenges

To avoid repeating clinical trial failures of the past, a large body of work has been dedicated to characterizing the safety of gene therapy approaches before and during clinical trials. Specifically, immunogenicity, transduction toxicity, and genotoxicity are being studied. Immune response against viral vectors, specifically AAV, have been extensively studied and clinical studies are provided valuable insights but many questions remain [327]. The Wilson group recently identified severe toxicity in pigs and nonhuman primates injected with AAV serotype hu6 (a variant of AAV9) at a high dose similar to the dose used in clinical studies for spinal muscular atrophy [328].

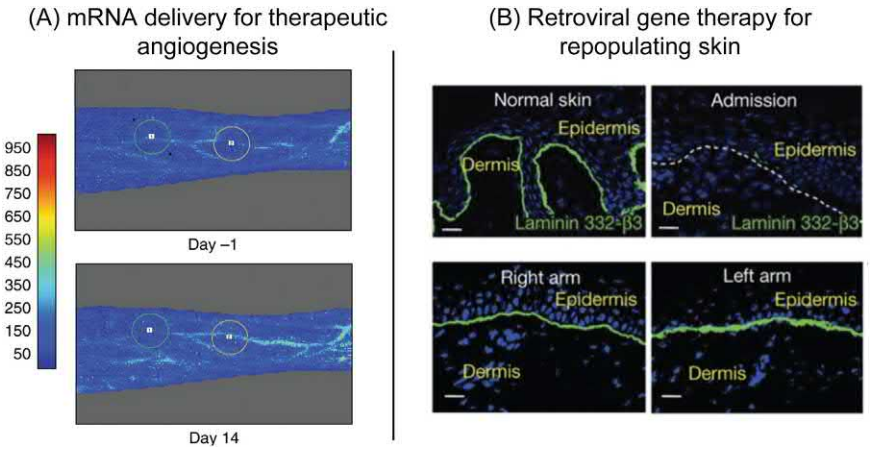

FIGURE 29.9 Clinical trial results for gene delivery and tissue engineering. (A) VEGF-modified mRNA was used to increase angiogenesis and perfusion in patients with type 2 diabetes shown here by laser doppler fluximetry. (B) A single-patient trial restored Laminin 332 expression by retroviral transduction of autologous keratinocytes with near complete restoration of the epidermis. In this image, green indicates Laminin 332 staining, which is absent after admission of the patient (upper right image) but complete restored at follow up (lower images). *Reprinted with permission from Hirsch T, et al. Regeneration of the entire human epidermis using transgenic stem cells. Nature 2017;551:327–32, doi:10.1038/nature24487; Gan LM et al. Intradermal delivery of modified mRNA encoding VEGF-A in patients with type 2 diabetes. Nat Commun 2019;10:871, doi:10.1038/s41467-019-08852-4.*

The authors noted dorsal root ganglia sensory neuron lesions necessitating euthanasia of the piglets and transaminase elevation in primates with one of three primates euthanized for liver failure. The authors suggest that the toxicity is caused by the transduction and not an immune response to transgene or capsid [328]. Genotoxicity can develop from unwanted genetic changes induced by viral vectors or genome editing. Unintended integration upstream of oncogenes resulted in cancerous transformation as seen in a previous clinical trial for X-SCID [77]. New generations of SIN retrovirus have avoided cancerous transformation in more recent clinical work [329] while other modifications can change the integration profile to reduce integration into actively transcribed genes. AAV has a low rate of integration which has raised some genotoxicity concerns in the past that have been controversial [330–333]. In the context of genome editing, undesired genome modification on- and off-target may potentially lead to genotoxic outcomes. Previous work has shown undesired genome modifications at off-target locations in the genome or at the target site inducing unwanted changes including vector integrations [334], large deletions [335], or large duplications [336]. In one case, the large duplication created a negative immune phenotype in mice [336]. Efforts should be made during clinical development to characterize genotoxicity including monitoring activation of oncogenes and clonal expansion of transduced or gene-edited cells.

Outlook

Three decades of laboratory-based gene and nucleic acid delivery have provided compelling arguments for continuing the refinement of vehicles and mechanisms for improving the safety and efficacy of gene manipulation. Recent advances in vector design have yielded vectors with improved systemic distribution and spatiotemporal control for gene expression which has propelled gene therapy into clinical evaluation for numerous conditions. Carrier-free technologies are also rapidly emerging based on innovative, highly stable nucleic acid chemistries, especially for applications where siRNAs are deployed in conjugate formats where targeting or hydrophobic modifications contribute to cell penetration. Relevant to tissue engineering, there are sharp contrasts among the design features for systemic versus local delivery, acute versus sustained activity, regulated versus constitutive expression, and extrachromosomal versus chromosomal localization. Thus the delivery criteria vary by application and the etiology of the disease.

Numerous challenges remain to optimize therapeutic potential for delivery of nucleic acids including increasing efficiency and ensuring safety of delivery strategies. Preclinical and clinical work will seek to characterize and avoid immune response to carriers, genetic cargo, and expressed genes. More work to detect cellular toxicity and genotoxicity will be needed. Ongoing clinical work will seek to address these concerns and characterize long-term safety and efficiency in patients.

In the coming years, we expect vigorous progress in increasing efficiency of delivery to new cell types and tissues. Tissue engineers will continue to develop new strategies for controlled gene delivery. Combined with advances in virology, new chemistry approaches, increased capacity for high-throughput screening, and

new technologies for specific DNA editing, the future is bright for gene delivery to improve tissue regeneration. Ongoing clinical trials in gene therapy will pioneer the frontier of genetic medicine brining us closer to revolutionary improvements in tissue engineering.

Acknowledgments

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Part Eight

Breast

Chapter 30

Breast tissue engineering: implantation and three-dimensional tissue test system applications

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Introduction

The chance of a woman developing breast cancer in her lifetime is approximately 13% [1]. It was estimated that in 2020 there would be approximately 276,000 new breast cancer cases and 42,000 breast cancer–related deaths in the United States [1]. The chance of breast cancer–related death in women is approximately 2.6%. Fear of disfigurement due to mastectomy or lumpectomy and limitations of breast-conserving options precipitated interest in tissue-engineering breast reconstruction options. Similarly, the impact of the disease, both psychological and physical [2,3], and the limitations of traditional two-dimensional (2D) bioassays used to understand, combat, or prevent the disease have driven the interest in engineering three-dimensional (3D) tissue test systems.

This chapter summarizes the various cell types that may be useful for breast reconstruction, the polymers that are being used or explored for use in breast reconstruction, and the limitations/advantages of specific animal models that allow one to test new tissue-engineering approaches. Avenues to potentially promote the vascularization of engineered tissues are discussed, as the major limitation in engineering large tissue volumes is the inability to deliver nutrients and remove waste products once the tissue is implanted. Finally, the chapter overviews the concept of tissue engineering to create benchtop breast tissue test systems that may be able to aid in developing breast cancer therapies and preventatives.

Breast anatomy and development

The breast is a dynamic organ that evolves constantly throughout a woman's lifetime. This tissue comprises multiple cell types that actively interact with each other [4]. Microenvironmental signals are key to the developmental processes of the breast throughout maturation [1]. The complexity of the breast must be considered when investigating strategies for engineering breast tissue; however, understanding of the many interactions that occur between the stromal cells and epithelial cells in the breast tissue remains limited. Evidence indicates that paracrine molecules produced by stromal cells likely determine the success or failure of tissue-engineered solutions for breast tissue repair [5]. To better understand the development of breast cancer, it is important to first review the anatomy of the breast and the structures and functions of the tissues (Fig. 30.1). The breast structure is located on top of the pectoralis muscle, which is located on top of the rib cage. Each breast contains 15–20 lobes that comprise 20–40 lobules [6]. It is inside these lobules that the mammary glands responsible for milk production are found [7]. The lobules are connected together through ducts; the milk is collected in the ducts and then flows out through the nipple. The space between these structures is filled with fat and fibrous tissue, the ratio of which determines breast density. A high concentration of lymphatic vessels and lymph nodes is found throughout breast tissue; these structures facilitate the flow of lymph, which comprises white blood vessels called lymphocytes and a fluid from the intestines called chyle, which contains proteins and fats [8]. The lymph flows to the nearby lymph nodes located in the underarm, above the collar bone, and behind the breast bone [7]. Blood vessels are also present to carry blood around the tissues to provide nutrients to the cells. The size and shape of the breast is determined

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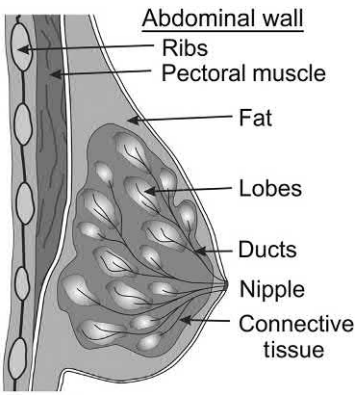

FIGURE 30.1 Breast physiology.

by the skin envelope and the adipose tissue surrounding the connective and glandular tissues. The firmness of the breast mound is dependent on the number of adipose clusters located within the breast, with higher adipose content resulting in a softer breast mound. The deep fascia and a thin layer of loose connective tissue are located between the breast and the pectoralis muscle; the connective tissue allows the breast to move freely over the deep fascia. The breast is attached to the skin through suspensory ligaments, also termed Cooper's ligaments, which provide additional support and contribute to the shape of the breast mound [9].

Several changes occur in the breast as a woman goes through puberty and menopause. During puberty, hormones released by the ovaries and pituitary gland cause the tissue to grow and the ducts to expand, forming mature ductal structures. While the structures are completely formed, they do not become fully active until pregnancy, when the lobules grow and begin producing milk. During menopause, when hormones are no longer produced by the ovaries, the lobule count in the breast decreases, and those lobules that remain shrink in size. This change leads to a lower breast density, since the ratio of dense, fibrous tissue to adipose tissue decreases. For this reason a woman's breast is typically denser before menopause than after [9].

Breast cancer diagnosis and treatments

Historically, breast cancer was not detected at a very early stage and therefore was treated with radical mastectomy, or removal of the entire breast, underlying pectoral muscles, and axillary lymph nodes. As breast cancer detection methods improved, however, breast cancer was discovered at earlier stages, allowing modified radical mastectomies or removal of small tumors. Randomized prospective clinical trials, conducted over 18 years comparing less deformative techniques, demonstrated equivalent survival rates to modified radical mastectomy [10]. These studies demonstrated that, for most women with small breast tumors, simple excision (lumpectomy) of the breast cancer, sampling of the axillary lymph nodes, followed by radiation provides a similar outcome to a radical mastectomy. A more recent study [11] of >5000 women with early stage breast cancer showed, over an 18-year period, no difference in survival rate for women undergoing breast-conserving surgery (e.g., lumpectomy) versus those undergoing mastectomy. However, breast-conserving surgery coupled with radiation treatment resulted in significantly enhanced survival rates in 10 years. Because the time span of the data studied is so great, the information should be further examined to determine correlation, if any, between age and treatment and if treatment options change over time with advances in knowledge and technologies.

Different treatment options can be used in combination to maximize the desired outcome. Breast cancer patients have therapeutic options, such as chemotherapy, radiation, and hormone therapy, and/or surgical options. Most often the treatment will be a combination of surgical and therapeutic options; treatment is determined on a patient-by-patient basis since individual cases of breast cancer have distinct characteristics. The treatment option is chosen based on the stage of the cancer (e.g., in situ or invasive), size of the tumor, health condition of the patient, and several other factors.

There are two main surgical procedures, mastectomy and lumpectomy. The progression of the breast cancer is the main determinant of the procedure. If the cancer has spread beyond the tumor mass formation, then a mastectomy, or removal of the entire breast, is recommended. If the cancer has not progressed outside the initial tumor mass, then a lumpectomy, or removal of the cancerous mass, can be performed followed by the use of an adjuvant therapy, most often radiation [12].

A mastectomy involves the removal of the interior of the breast mound, the nipple, the areola, as well as a wide margin of tissue around the incision. Prior to surgery, the extent of the axilla tissue that must be removed in order to remove the cancerous tissue is determined. Cancer cells most easily spread through the lymphatic system, so the progression of the cancer can be determined by examining the lymph nodes around the cancerous breast [12]. Because of the ease of travel through the lymphatic system, it has become common practice to remove some of the surrounding lymphatic vessels and nodes.

Breast reconstruction

There are extremely limited cosmetic surgical procedures available for lumpectomy patients. In select instances, tissue flaps or fat grafts may be implanted; however, the surgical processing and implantation technique is paramount

to the viability of the transplant, where deviation from established procedures can result in calcification of the breast tissue [13]. Following a mastectomy, patients are given the option of undergoing breast reconstruction surgery. The type and timing of reconstruction is determined first by the physical limitations of the patient and then by preference. An option that has grown in popularity is breast reconstruction immediately following mastectomy [14]. Previously, it was thought that reconstruction should be delayed to prevent any possible interference with an adjuvant therapy. However, benefits to immediate reconstruction include one surgery and hospital stay, better psychological outcomes, and improved aesthetic results. Risks involved with this combination surgery include extended surgical time and an increase in complexity of the procedure [14]. The risk and rewards of undergoing such a procedure are evaluated on a patient-by-patient basis. There are two primary types of tissue implants for breast reconstruction: synthetic and autologous.

Synthetic implants

Generally, synthetic implants are simpler and require less surgical time, but the results are not as aesthetically satisfactory. The simplest reconstruction is a silicone breast implant, which is a silicone gel−filled or saline-filled silicone bag that is implanted in the submuscular position beneath the removed breast mound. In some instances the void volume may be increased by the progressive inflation of a tissue expander [14] prior to the placement of a silicone breast implant. Because an implant is a foreign body, it may trigger a substantial inflammatory response, resulting in fibrosis, thickening, capsular contraction, and an unnatural shape and tactile quality. Also, implants may leak and require replacement. Silicone-filled implants are now rarely used due to fears of possible complications caused by the leakage of silicone.

Tissue flaps

Autologous breast reconstruction relies on the use of the patient's own tissue, is more complex, requiring more extensive surgery and a longer recovery time, but the results are much more natural and aesthetically pleasing than those of prosthetic implants. There are several primary tissue retrieval sites, including the abdomen, the back, and the buttocks or thigh. The transverse rectus abdominis myocutaneous (TRAM) flap, located in the abdomen, is surgically excised, including fatty tissue, abdominal wall skin, often with the blood supply network intact, and is molded into the breast mound. A TRAM flap procedure requires that an additional surgery be performed to reconstruct the nipple−areola and to improve the shape of the reconstructed breast mound; a weakening of the abdomen as well as contour abnormalities of the abdomen can occur after this procedure [9]. The latissimus dorsi flap is removed from the back in a similar but less involved manner than removal of the TRAM flap. Disadvantages of this flap procedure include susceptibility to atrophy and lack of patient-to-patient tissue volume consistency. Muscle-free flaps from the buttock (gluteal) or thigh (tensor fascia lata) may also be used; in some instances a muscle sparing procedure may be used, wherein only fat/skin is transplanted. Adipose tissue matrices, devoid of lipids and cells, have been investigated for use in soft tissue defect repair [15].

Cell transplants

Some of the very first studies performed regarding adipose tissue replacement used a method called autologous fat transplantation. This methodology simply involved harvesting adipose tissue from a location into the patient and transplanting that tissue to the breast tissue void. This procedure was completed without a scaffold in place to guide the shape of the tissue replacement. The results of this type of procedure were very poor, with 50%−70% reduction in volume due to the resorption of the grafted tissue. Resorption occurred because adipocytes are anchorage dependent and require a scaffold to survive. In addition, the adipocytes found in the tissue graft were terminally differentiated and therefore could not proliferate [9]. After many failed cell transplantation attempts, it was suggested that a scaffold was required for proper breast tissue replacement. However, while scaffolds became a subject of significant research activity, interest in lipofilling continued, and studies raised awareness of both the inhibitory and stimulatory potential of lipofilling and underscoring the criticality of the extraction and implantation processes/techniques in free fat transfer [16]. A review of clinical cases suggests that injection of a fat bolus can lead to fat necrosis, oil cysts, and long-term inflammation and calcification. The review further suggested that the better results are achieved by injecting fat diffusely as tiny aliquots throughout the target site [13]. Improved methods in cell harvesting are needed to increase the yield of viable fat cells. Cryopreservation technologies must also advance to allow later serial injections, obviating the need for repeated fat removal procedures.

The cellular material used for transplantation is obtained from lipoaspiration, a process that can damage and lyse the cells. Researchers are developing means with which to treat lipoaspirate, for example, it may be washed in a polyoxamer, which is thought to stabilize the cellular membranes of damaged adipocytes and lend greater stability for implantation and graft survival. Preliminary studies suggest that the treatment can increase implant viability post implantation [17].

Cellular scaffolds

Breast tissue engineering is another reconstructive option, beyond that of synthetic or autologous implants. Breast tissue engineering may involve cellular or acellular scaffolds. Much of the current research is still focused on traditional approaches of ex vivo cell expansion; however, due to financial, regulatory, and logistical obstacles, more focus must be placed on "just-in-time" delivery options that do not incorporate cell expansion ex vivo. Currently, viable breast tissue-engineering technologies have not been translated from the laboratory to the clinic due to several existing challenges related to cell culture, scaffold type, and animal model selection that affect our ability to build a vascularized tissue that maintains volume in the long term.

Cell types and related challenges

Many issues must be considered when choosing which cell type(s) should be used in human breast tissue engineering. The first consideration is that there is substantial variability in the size, shape, and consistency of the breast. The breast changes over time, with a tendency for breast parenchyma (glands and ducts) to involute or regress as a woman ages, particularly after menopause, and be replaced by fat [18]. Also, comparing breast tissue among women of any given age, there is considerable variability in the size, shape, tactile, elastic, and tensile characteristics of the tissue. The tensile and elastic characteristics of the breast are influenced by three major factors:

1. the amount and quality of fat within the breast
2. the amount and quality of glandular and ductal tissue in the breast
3. the mechanical characteristics of the fibrous support structures of the breast (Cooper's ligaments)

The creation of a functional breast with lactational ability is not needed and, in fact, may add to a woman's breast cancer risk by introducing mammary epithelial cells that may be predisposed to cancer development. The major, immediate goal of breast reconstruction is to produce a breast mound with all of the aesthetic properties of a normal breast.

Normal breast tissue comprises adipocytes; however, these lipid-laden fat cells (adipocytes) are terminally differentiated and will not divide further in vivo or in vitro. Indeed, studies have shown that if not maintained in a 3D culture environment, these differentiated cells will likely dedifferentiate and become fibroblastic [5]. Hence, the use of adipocytes requires harvesting fat in the exact volume required for the construct [19–21]. In addition, the majority of mature fat cells in a lipoaspirate sample rupture [8,9,21]; accordingly, measures must be taken to compensate for or prevent this loss.

Preadipocytes may be used for engineering soft tissue [22,23] as these cells are not susceptible to retrieval damage and can potentially be expanded in culture. Preadipocytes are similar to fibroblasts in structure and possess the ability to expand in culture [24]. Several studies have shown ways of inducing differentiation into adipocytes. These cells can be successfully harvested and isolated from sites such as the subcutaneous tissues or the omentum. Investigations of autologous preadipocyte implantation with a sheep model have been promising [25]. Technologies are being developed to allow simple harvesting and fast isolation of preadipocyte cells for immediate reimplantation into the patient [26]. Studies in mice have shown that the incorporation of "adipose-derived regenerative cells" in fat grafts decreases cell apoptosis and increases expression of growth factors [27]. Induced pluripotent stem cells (iPSs), that is, cells derived from skin or blood cells and genetically reprogramed to an embryonic stem cell–like state, have been tested for adipogenic potential; in one specific instance assessing reprogramed human dermal fibroblasts, the iPSs were found to have similar lipid accumulation and transcription of adipogenesis-relevant markers as those found in two human embryonic stem cell (hESC) lines [28].

Possible other cell types include smooth muscle cells, fibroblasts, skeletal muscle, and elastic cartilage. Fibroblasts contribute greatly to the support structure of the breast by laying down bands of collagen that connect the breast tissue to the skin and to the pectoral muscle, as well as helping maintain the overall shape. The density and firmness of the breast is determined primarily by the glandular epithelium and ductal structures, where tissues that have similar tactile and elastic properties are almost exclusively muscle. Smooth muscle cells can be readily isolated from a number of organs and expanded in culture; implantation of smooth muscle-containing polymers can lead to the reformation of significant tissue masses, with reorganization of the smooth muscle tissue into appropriate 3D structures [29]. Muscle myocytes can also be greatly expanded in vitro, and have been demonstrated to reform functional tissue masses under appropriate conditions [30,31]. However, it remains to be demonstrated that smooth or skeletal muscle myocytes will maintain a tissue mass over long periods of time without neural stimulation. An alternate approach might even be to incorporate chondrocytes in an engineered breast tissue. Elastic cartilage has many of the mechanical properties of glandular breast tissue that are potentially important for tissue engineering of breast (e.g., elasticity). Chondrocytes can be expanded in culture and are able to survive in low oxygen tensions. Chondrocytes have been used extensively in tissue engineering to engineer a variety of tissue constructs both in vitro and in vivo [32–34].

However, the more commonly studied cell types for breast tissue engineering include human bone marrow–derived mesenchymal stem cells (hBMSCs) and human adipose-derived mesenchymal stem cells (hAMSCs) [35]. Both cell types have the ability to differentiate into adipocytes when introduced into an adipogenic differentiation medium. The hBMSCs are harvested from the bone marrow, through a relatively painful procedure, which lessens their clinical relevance, while hASCs can be harvested in large volume from adipose tissue obtained via biopsy or liposuction [35]. Recognizing that regeneration may most effectively occur in situ with cues from the native tissue, cell types such as mesenchymal stem cells and embryonic stem cells have rapidly become the most popular cells for breast tissue-engineering research. hESCs are readily able to differentiate into adipocytes [35]; however, a great deal of work will need to be done to ensure terminally differentiated cells, which once transplanted, do not have the potential to form a tumor mass. The implantation of embryonic stem cells has the potential to result in teratoma formation in vivo [36,37]. Studies in mice have shown that the formation may be site dependent and exacerbated in the presence of specific biomaterials [38].

Standard cell isolation and expansion protocols must also be developed; critical steps influencing successful outcomes will include aseptic technique in the harvest, routine quality control testing of all cultures, long-term cell storage, and on-site operating room handling. It may also be possible to isolate all the cell types required for breast tissue engineering from a single tissue source; furthermore, it may be advantageous to retrieve tissue isolates rather than cellular isolates [39]. For example, fat, in addition to adipocytes, contains a large vascular network, composed primarily of capillary endothelial cells and some vascular smooth muscle cells as well as a collagen stromal structure produced by fibroblasts [40]. Hence, multiple cell types or cellular aggregates (tissue isolates) can potentially be obtained from this tissue. In attempting to produce fat tissue in vivo, it may be beneficial to expand the cellular components of fat without isolating each component, as the complexity of the mechanisms may be crucial and impossible to rebuild from cellular blocks. Given the complexity of tissue, it is most likely that a mix of cells, with respect to maturity and type, will result in the most promising tissue-engineering solution.

Scaffolds

An important consideration in engineering breast tissue is the scaffold selection. Selection of a material chemistry and form to use as a scaffold depends on the purpose and characteristics of the tissue that is being replaced as well as the physical characteristics and health of the patient. Scaffolds may be fabricated to induce tissue integration or they may be developed to house or attract cells which, in turn, assist in inducing tissue integration. Several key characteristics make a biomaterial suitable for tissue-engineering application. First, with in vivo endpoint, favorable biomaterials must be absorbable or degradable and therefore facilitate new tissue integration with native tissue over time. The shape and texture of the material does not have to resemble that of natural tissue, but it should induce growth of new breast tissue resembling native tissue, that is, be soft and pliable. Cellular affinity is another important aspect of a biomaterial, whether considering in vivo or in vitro endpoint. The biomaterial must interact favorably with cellular components without negative impact (e.g., the material cannot be tumorigenic or toxic). Some element of porosity or surface texture is helpful and potentially allows cellular ingrowth into the material and/or transfer of nutrients and waste products, the establishment of a vascular network into the biomaterial scaffold [41], and/or differentiation of multipotent cells [42].

Synthetic materials

Aliphatic polyesters of polyglycolide (PG) and polylactide (PL) are well-characterized synthetic biodegradable polymers that are clinically familiar to biomedicine and are familiar also to tissue-engineering researchers [43]. PG is highly crystalline and has a high melting temperature and low solubility in organic solvents. PL is more hydrophobic than PG due to the presence of a methyl group. PL has a low water uptake and its ester bond is less susceptible to hydrolysis, due to steric hindrance by the methyl group. Therefore PL degrades more slowly and has higher solubility in organic solvents than PG. Copolymers of PL and PG can be readily synthesized; their physical properties are modulated by the ratio of glycolic acid to lactic acid. Often incorrectly named in the literature, PL and PG are synthesized by ring-opening polymerization, while the lower molecular weight polylactic acid and polyglycolic acid are synthesized by step growth. The latter are in the order of thousands of Daltons and are limited to use in structures with low mechanical demands, such as films and spheres [44]. Aliphatic polyesters can be readily processed into various physical forms appropriate for tissue-engineering applications. A number of techniques have been proposed to generate highly porous scaffolds, including solvent casting/particulate leaching [45], phase separation [46], emulsion freeze-drying [47], fiber extrusion and fabric formation [48], and gas foaming [49,50].

Polyethylene glycol (PEG) scaffolds [51] are readily modified by degradation and adhesion peptides and thus have been the subject of preadipocyte studies. The combination of adhesion and degradation features appears to allow

the highest adhesion and proliferation of preadipocytes. Nondegradable polymers have also been examined for tissue reconstruction applications. For example, in vitro studies have been conducted with fibronectin-coated polytetrafluoroethylene (PTFE) scaffolds. Human preadipocytes have been shown to successfully attach, proliferate, and differentiate into adipocytes on the PTFE scaffolds [52]; however, these nondegradable scaffolds are likely better suited for 3D in vitro tissue test systems.

A number of other synthetic polymers could be used to fabricate scaffolds for breast tissue reconstruction, including polycaprolactones (PCLs), polyanhydrides, poly(amino acid)s, and poly(ortho ester)s [53]. PCL is an aliphatic polyester, a semicrystalline polymer with high solubility in organic solvents, a low melting temperature, and a low glass transition temperature (T_g). The degradation rate of PCL is much slower than PG or PL; because of the low T_g, PCL has a flexible, sticky quality that can be advantageous in a scaffold. PCL has been tested in scaffold form in animals [54,55] and is used clinically in orthopedic applications. Polyanhydrides are usually copolymers of aromatic diacids and aliphatic diacids. These materials degrade by surface erosion, the rate of which can be controlled depending on the choice of diacids [56]. Poly(amino acid)s have been studied due to their similarity to proteins and widely investigated for use in biomedical applications such as sutures and artificial skin [57]. Poly(amino acid)s are usually polymerized by ring opening of *N*-carboxyanhydrides; versatile copolymers can be prepared from various combinations of amino acids. However, due to the low solubility and limited processability of poly(amino acid)s, "pseudo"-poly(amino acid)s were developed [58]. It has also been reported that poly(amino acid)s containing L-arginine, L-lysine, or L-ornithine cause endothelium-dependent relaxation of bovine intrapulmonary artery and vein, and they stimulate the formation and/or release of an endothelium-derived relaxing factor identified as nitric oxide [59]. Poly(ortho ester)s are biodegradable polymers, which degrade by gradual surface erosion and have been investigated for controlled drug delivery.

Naturally derived materials

Naturally derived polymers have been used for adipose tissue engineering; scaffolds produced from these materials typically are hydrogels or structural forms such as mesh, sponges [9], or beads. Investigations have been conducted, for example, using Matrigel (reconstituted basement membrane of mouse tumor) and fibroblast growth factor 2 (FGF-2) to induce in situ adipogenesis. Matrigel consists largely of type IV collagen, laminin, and perclan and, although a highly variable material with many ill-defined components, it is considered the gold standard in cancer cell biology benchtop studies. Preliminary small animal studies have demonstrated the migration of native preadipocytes as well as endothelial cells into Matrigel when this material is injected into subcutaneous tissue [9,41,60].

Hyaluronic acid (HA) is a natural component of the extracellular matrix of many tissues. HA comprises repeated sequences of glucuronic acid and acetylglucosamine; this material is susceptible to enzymatic degradation via hyaluronidase. Simple modifications have been made, such as cross-linking the chains to form insoluble hydrogels [61]. In its natural form, HA plays a role in enriching wound healing by promoting early inflammation and stimulating angiogenesis [62]. Hyaluronan benzyl ester (HYAFF 11) scaffolds are derived from HA that is esterified with benzyl groups at the glucuronic acid monomer. Researchers experimented with these sponges, seeding them with human preadipocytes and surgically implanting them into subcutaneous tissue of athymic nude mice. The sponges allowed good cellular penetration as well as the development of new vascular networks within the sponges. However, adipose tissue development remained sparse [63]. The researchers also compared collagen scaffolds to HYAFF 11 scaffolds in vivo and concluded that there was increased implant weight, adipose tissue formation, and distribution of cells in the HYAFF 11 scaffolds [64]. Hydrogels of HA have been prepared by covalent cross-linking with various kinds of hydrazides [65] and have been used in drug delivery [66].

Collagen is the best-known tissue-derived natural polymer and is the main component of all mammalian tissues, including skin, bone, cartilage, tendon, and ligament. Collagen has been used as a tissue culture or artificial skin scaffold due to its high cell affinity. However, collagen offers a limited range of physical properties, can be expensive [67], is highly variable, and can elicit a strong immunologic response. Chemical modification and incorporation of fibronectin, chondroitin sulfate, or low levels of HA into the collagen matrix can change cell adhesion [68]. In vivo comparisons of freeze-dried collagen scaffolds with HA sponges and nonwoven mesh, implanted in mice for 8 months, revealed a greater number of adipocytes in the hyaluronic sponges than in the nonwoven mesh. This difference was mainly attributed to the porous nature of the sponge, which allowed greater surface area for adipocyte cell distribution and growth [64,69]. Recent studies also have assessed the idea of creating a fibrovascular tissue bed or natural scaffold, using a preexpansion vacuum into which fat cells are transplanted [70].

Alginate is a naturally occurring hydrogel that can be easily formed into an injectable gel or beads but must be modified with a peptide sequence to allow cell attachment [25,71,72]. Interestingly, the human body does not contain

alginase, the enzyme that breaks down the alginate chain; hence, molecular weight is a crucial consideration for implantation as large molecular weight alginate chains will not be eliminated from the body. That is, the molecular weight of many alginates is typically above the renal clearance threshold of the kidney [73]. Alginate chains are bound together with divalent ions that migrate in areas of divalent ion deficiency, causing uncontrolled dissolution. To address this point, hydrolytically degradable, covalently cross-linked hydrogels derived from alginate were developed [74]. Specifically, polyguluronate blocks with molecular weight of 6000 Da were isolated from alginate, oxidized, and covalently cross-linked with adipic dihydrazide. The gelling of these polymers could be readily controlled, and their mechanical properties depended on the cross-linking density. It was also demonstrated that alginate gel degradation can be readily regulated by controlling the molecular weight distribution of the polymer chains in the gels, and their susceptibility to hydrolytic scission by partial oxidation [75].

Other materials have found limited, preliminary use in breast tissue engineering. Chitosan is relatively biocompatible and biodegradable [76,77], making it useful for breast tissue engineering [78] and wound healing [79]. Chitosan is abundant and easily derivatized by coupling molecules to the amino groups [79,80] and has shown early success in murine studies focused on injectable breast tissue-engineering systems [39]. Fibrin glue has been used as an adipocyte scaffold and, in small animal studies, has facilitated maintenance of adipose tissue up to 1 year after implantation [81]. Fibrin is a blood-based product, a characteristic which has slowed its translational appeal in the United States.

Therapeutic scaffolds

Some scaffolds provide more than a simple matrix on which cells will grow. Some scaffolds have an incorporated therapeutic agent such as a drug or growth factor and are termed therapeutic scaffolds. The concept behind these scaffolds is that the therapeutic agent incorporated in the scaffold is released as the cells remodel the scaffold during cell growth and proliferation. The therapeutic agent therefore has a direct effect on the surrounding tissue where the scaffold is placed; accordingly, the use of growth factors and the stimulation of cell growth must be carefully evaluated in breast cancer–related reconstruction, to avoid facilitating the cancer process.

Indeed, therapeutic agents can affect the tissue surrounding the implanted scaffold. An example can be seen in a study where the therapeutic agent, angiogenin, was incorporated into a scaffold [82]. Angiogenin is a drug that has been shown to promote neovascularization, so the intent of its incorporation was to help promote the growth of new vasculature throughout the scaffold, increase the chances for the success of an implanted tissue replacement, and improve the overall outcome of the procedure. The investigators subcutaneously implanted these scaffolds into rabbits and, after 28 days, the scaffolds were excised. The study showed that scaffolds with incorporated angiogenin had increased neovascularization. Other therapeutic scaffolds have been designed; for example, a collagen/chitosan/glycosaminoglycan scaffold was assessed [83]. The agent for this therapeutic scaffold, transforming growth factor-beta 1 (TGF-β1) targeted cells grown on the scaffolds rather than the surrounding tissue. Accordingly, TGF-β1 was incorporated into chitosan microspheres that were embedded into the scaffold.

A final example of a therapeutic scaffold is one in which the agent targets and neutralizes a specific type of cell found in the surrounding tissue, such as a cancer cell. Researchers developed, for example, scaffolds with nanoparticles containing emodin, an anticancer drug [84]. These scaffolds were intended to fill a site where a cancerous tumor was removed from the breast. The concept of this scaffold is that as cells proliferate on the scaffold and remodel it, the emodin contained within the nanoparticles is released and neutralizes cancerous cells in the surrounding tissue. These scaffolds were implanted next to the mammary fat pads of nude mice in which cancerous cells had been injected. The results of this study indicated that the size and number of the tumors next to emodin-loaded scaffolds were reduced compared to those next to scaffolds without emodin.

Injectable scaffolds

Early scaffolds took forms such as foams or mesh; they required implantation via an open surgical procedure. However, the reality of the transport and surgical limitations of large volume implants led to the development of injectable composite scaffolds, comprising a “solid,” absorbable fraction of injectable size in a degradable carrier gel [85]. Injectable scaffolds can be combined with cells and surgically implanted in a minimally invasive manner, including by syringe, catheter, or endoscopic needles [86,87]. These injectable materials take forms such as gels, beads, or composite gels. Examples of injectable scaffold chemistries that have been assessed for breast tissue engineering include alginate, chitosan, HA, collagen, polyanhydride, degradable PEGs, decellularized adipose tissue, small intestinal submucosa, and blends thereof [15,39,85,88,89]. While gels support cell growth and readily conform to a defect, they typically do not support the necessary functions of anchorage-dependent cells and are therefore useful as carriers but not as scaffolds in breast tissue engineering. Beads and composites (which contain beads or other filler), in contrast, are amenable to breast tissue engineering.

Combination scaffolds

It is likely that a combination of biomaterials, with respect to chemistry and form, will result in the most promising tissue-engineering solution. Scaffold combinations that use two or more types of materials can be used to help combat and overcome the weaknesses and shortcomings of one material. For instance, a material that has excellent cell attachment characteristics but is not very durable can be combined with a more durable material, hopefully resulting in a scaffold that is both durable and able to support cell growth.

One of the first combination breast tissue-engineering scaffolds was an injectable composite comprising beads in a delivery gel [85]. Indeed, cellular constructs that are approximately 500 μm in thickness or less, once implanted, may optimize diffusional transport of nutrients to the cells while each small cell-polymer unit becomes vascularized. Accordingly, these injectable composites were developed specifically to allow trafficking and infiltration of blood vessels, nutrients, waste products, other factors, and cell types, within the discrete portions of the scaffold (i.e., between beads) [39,85,90]. The beads, or a fraction of the beads, may be selectively loaded with appropriate factors to induce tissue growth or prevent abnormal tissue growth. The gel is degradable and facilitates delivery of the composite through a needle and also allows the composite to conform to and fill an irregular defect site. The gel may be loaded with factors for release on degradation. A variety of studies have been conducted to demonstrate the modularity of an injectable composite approach [39,72,85,91,92].

It is unlikely that there is a one-size-fits-all biomaterial chemistry or form; accordingly, there is interest in a wide range of combination scaffolds. In one study, gelatin sponges, PG mesh, and monofilament polypropylene mesh were used to construct 3D scaffolds of predefined shapes on which hAMSCs were grown [93]. The scaffolds were made of an outer polypropylene mesh pocket that contained gelatin sponge cubes and PG mesh. Gelatin is an attractive scaffold for cell growth and attachment and can be molded into a desired shape but rapidly loses its dimensional stability over time. The PG mesh was used to increase the surface area available for cell attachment. Polypropylene mesh was used because of its ability to maintain dimensional stability after being implanted into the body. These scaffolds were seeded with a high-density hAMSC suspension and cultured for 2 weeks. Subsequently, the scaffolds were implanted into the backs of nude mice for 2 months and then excised for analysis. Analysis showed that the scaffolds contained new adipose tissue as well as neovascular structures. The gelatin cubes, as well as the PG mesh, were completely absorbed by the body, but the outer polypropylene mesh retained the predefined dimensions; the neovascular structures may have simply been the transient part of the normal foreign body response.

Strategies to enhance the vascularization of engineered tissue

A critical challenge to engineer breast tissue, or any tissue of significant thickness, remains the development of a vascular network to support the metabolic needs of the engineered tissue and integrate it with the rest of the body. Adipose tissue is highly vascularized, with a resting blood flow two to three times higher than that of skeletal muscle. The presence of vascularized networks in natural metabolic organs results in short diffusion distances between the nutrient source and the cells [94], and these vascular networks must be created in engineered tissues as well. Nutrient diffusion in vivo is constrained to a distance of approximately 150 μm. Most metabolically active cells that are located further than this distance from a nearby capillary are subject to hypoxia. Thus the success of any large engineered tissue hinges on its blood supply. The important interrelationship between preadipocytes and endothelial cells was demonstrated in hypoxic culture experiments. Frye and coworkers [95] exposed cell cultures of pure preadipocytes, as well as mixed cultures of preadipocytes and microvascular endothelial cells, to hypoxic conditions (5%−2% O_2) and found that preadipocytes cocultured with microvascular endothelial cells had higher viability than cultures consisting of preadipocytes alone.

Several general approaches have been taken to date to promote angiogenesis in engineered tissues; however, none provide a robust, consistent solution to this complex problem. Bland and coworkers [96] provided a detailed review of approaches to combat hypoxia in tissue-engineered systems. In short, this problem has been unsuccessfully addressed in the long term by incorporating endothelial cells or angiogenic factors in tissue-engineered implants. Concerns about blood vessel promoting factors are high when targeting solutions for breast cancer patients. The composite injectable systems do provide the option of gradually building smaller volumes of tissue over time to collectively build a large volume [97]. Many studies highlight angiogenic response at short time points, suggesting that the foreign body response provides the necessary vascularity [98,99]. Indeed, while this is the case in the short term (and at later defined intervals, as the material is further degraded or absorbed and elicits further response), the vascularity is temporary only. Blood vessel ingrowth occurs slowly with this approach, will likely not be sufficient to engineer large tissue volumes, and will likely subside with termination of the foreign body response. Other approaches attempt to actively

modulate the vascularization process by either delivering angiogenic molecules or blood vessel forming cells (e.g., endothelial cells) to the site at which the tissue is being engineered. Prevascularization, either in vitro or in vivo, has also been proposed and investigated by numerous groups, but the problem of integrating newly developed vasculature with host tissue remains unsolved. In addition, for reconstruction following lumpectomy or mastectomy, there is concern of igniting rapid vascular growth and supporting cancerous cell growth [100].

Microstamping using nano-fluid chambers is one research approach of interest in liver tissue engineering [101], where the long-term goal is to develop a 3D vascular bed ex vivo that could be anastomosed to the host vasculature to support cellular engraftment. This approach could also be developed for breast tissue engineering. Another approach involves the direct association of an implant with a preexisting blood supply. Experiments with nude mice were performed, in which silicone molds packed with PG fibers were sewn to the inferior epigastric blood vessels. These silicone molds were injected with a combination of Matrigel and FGF-2. In situ adipogenesis was demonstrated over a 4−20-week time course. Direct application of a vascular pedicle to a construct is another promising approach. Prefabricated flaps have been created with vascular pedicles since the 1960s; vascular pedicles can be supplied in many different configurations. In general, however, conduits comprising an intact artery and vein fare better than those comprising a single vein alone [102,103].

Special considerations

The need for nipple reconstruction occurs after mastectomy. Traditional implants include free composite grafts, local tissue transfer, and prosthetic devices. Free composite grafts were initially used and created from autologous tissues such as the labia, inner thigh, cartilage (auricular or costal), the contralateral nipple, or the toe. Complications at the graft site made this technique less desirable. Local tissue flaps are the most popular option for nipple reconstruction. Commonly used techniques include the bell flap, the modified star flap, and skate flap [104−107]. Unfortunately these techniques can be hindered by flap necrosis and poor aesthetic results, including loss of nipple projection. In addition, the presence of underlying subcutaneous fat is important for bulking; this layer is not always sufficient. Tissue-engineering approaches include the use of tissue flaps coupled with acellular, naturally derived (collagen, extracellular matrix, etc.) matrices [108] and/or fat grafts.

Breast cancer modeling

As Savage stated, “Cancer is not a simple disease” [109]. A significant challenge to both the development of breast cancer treatments and fundamental understanding of breast cancer processes is the development of a tool, a model, which allows isolation and control of specific parameters of interest. The study of basic disease processes and treatments in a patient is generally very inefficient because of the number of confounding biological (and ethical) issues. In response to this challenge, researchers have proposed animal models, mathematical models, and benchtop test systems as the means to more effectively study breast cancer.

Animal models

It is important to first consider the application for which an animal study is planned before the selection of the animal model itself. Either the nude (nu/nu) or severe combined immunodeficiency (SCID) mouse model is used for the transplantation of human-derived cells [110]. These animals have compromised immune systems to the extent that they will often accept xenogeneic transplanted tissues, and these mice have been particularly useful for transplantation and immunologic studies of human tumors, bone marrow, skin, and other tissues [97,111,112]. SCID and nude mice are used to evaluate various polymer constructs with and without human cells in vivo, without the adverse effects of a major immunologic reaction. Furthermore, basic questions about human cells and polymers in vivo may be answered without launching human trials prematurely. Development of these models may not be straightforward since there are subtle differences between strains with respect to the acceptance of various tissues and growth of the implanted tissues in different sites (e.g., a tissue may grow in one mouse strain but not in another, or may grow in a subcutaneous site but not in an internal location). Even though mice are genetically altered for immunodeficiency, the particular genetic modifications can vary. These varying genetic modifications can cause varying levels of different hormones throughout the host body. Hormonal differences will result in adipose tissue development differences that will likely result in different biological reactions to the same implant. Several different genetic varieties of laboratory rats exist, which poses complications when attempting to compare studies [110]. Biologically, males and females of the same species have different hormone levels due to differences in development and maturation. In addition, gender-induced differences in hormones cause a difference in adipose tissue development which may result in different biological reactions to similar scaffold constructs [110].

Nude mice lack a T-lymphocyte response, while SCID mice lack both a T- and B-lymphocyte response. However, both types have natural killer cells that may interact with some transplanted materials. These mouse colonies must be monitored closely for changes in the immune status of

the mice that, at times, occur spontaneously. In addition, the human cells must be routinely screened for the human immunodeficiency virus, hepatitis, and mycoplasma before being transplanted into immunosuppressed mice to assure the safety of the animals and workers, and the validity of the experiments.

Another complicating factor is the size of the actual animal in question. Small size animals include animals such as mice and rats, while larger sized animals include sheep, domestic pigs, and cows [110]. When considering mammary tissue, the larger sized animals are more physiologically relevant to humans. This issue raises the question of applicability of relating animal model results to an expected human clinical trial outcome. Large animals can accommodate the same size implant as one that would be used in a human. Also, the internal anatomy of larger animals, such as the domestic pig, is much more similar to the human anatomy in regard to organ size and heart rate. It has also been shown that the histoarchitecture and hormonal control of the mammary glands of larger animal models is much more applicable to humans than those of rodents [113]. Studies have shown that bovine cells as well as human cells do not grow in the mammary fat pads of immune suppressed mice. This observation suggests that the fat pads of rodents do not provide an environment suitable for the proper growth of human or bovine mammary epithelium. It has also been shown that progesterone has a very different effect on the mammary tissue of mice when compared to that in larger animals, including humans. In mice, progesterone stimulates epithelial proliferation and ductal side branching, whereas progesterone has a limited effect on the mammary epithelium of larger animals and humans.

There has been excellent success using inbred female Lewis rats as a small animal model for the development of transplantable tissues with absorbable polymers such as PG, PL, poly(lactide-*co*-glycolide), and hydrogels such as alginate [114]. This model allows transplantation of cells between individuals without concern for immunologic rejection, which parallels the likely autologous nature of cell transplantation for breast engineering. In addition, the Lewis rat is larger than many other strains, allowing the testing of larger (1−2 cm) or multiple constructs in the same animal.

In evaluating larger animals as models for breast tissue engineering, animals with skin and subcutaneous tissues that are similar to humans are required in order to evaluate larger constructs subcutaneously. For this reason the same animal must be used as a tissue donor and recipient. Porcine skin and subcutaneous tissues are very similar to that in humans, but most pigs continue to rapidly gain weight throughout their lives, which makes monitoring implants very difficult. Sheep have very little subcutaneous fat and, depending on the location, have well-defined subcutaneous space for cellular engraftment. Hence, if new adipose tissue is formed there is a high probability that this is developed from the implanted cells. One of the few locations in the sheep where there are significant fat deposits is the omentum. Researchers have shown that preadipocytes isolated from the omentum can be expanded in culture, seeded onto porous alginate−RGD fragments [25], where they attach, proliferate, and spread onto the biomaterial surface. Cellular alginate−RGD fragments were subsequently injected into the nape of the neck of sheep to determine if new adipose tissue would form. Although the cells were autologous and not labeled with a tracking marker, there appeared to be de novo adipose tissue formation in the cell implant sites compared to the acellular biomaterial control sites.

Indeed, further investigation of breast tissue-engineering options within a large animal that has biological characteristics comparable to that of humans is required. The bovine mammary gland consists of the same anatomical structures and tissue types as that of the normal human breast [115]. Histological evaluation reveals that bovine mammary tissue is more similar to that of humans than is mammary tissue of traditional animal models such as mice and rats [90]. The ductal structures in the human and cow are surrounded by relatively dense stromal tissue [116,117] unlike the ducts in the mouse which are almost completely enclosed by adipocytes [118].

Table 30.1 summarizes the similarities and differences seen in mammary gland development in mice, humans, pigs, and ruminant animals such as sheep, goats, and cows. However, larger sized animals present logistical and financial issues. Large animals are much more expensive than smaller animals and fewer researchers are trained in, or have the facilities for, the proper care of these larger animals [113].

Breast tissue test systems

To gain better insight into the in vivo setting, 3D tissue test systems were first proposed as a new application of tissue-engineering methodology to create benchtop organs or tissues [119]. Subsequently, it became rapidly apparent that these benchtop systems could be used to model experimental clinical conditions or biological situations. Events that are notable in a 3D setting, that is, in vivo, may not be present in a 2D model, or vice versa [5]. Hence, 3D models are sought to probe 3D phenomena. Breast tissue test systems can be used to inform tissue-engineering reconstructive techniques, or to better understand and prevent the disease process through identification of biomarkers or decoding of complex pathways [120,121]. Many studies have been conducted to assess breast cell cocultures in 3D. The 3D material, that is, the scaffold, has enormous influence on cellular behavior and

TABLE 30.1 Comparative aspects of mammary gland development [113].

Attribute	Mice	Humans	Pigs	Ruminants
Morphology	Sparse ducts: alveolar	TDLU	TDLU	TDLU
Stromal histology	Adipose»connective	Intralobular, interlobular connective»adipose	Intralobular, interlobular connective»adipose	Intralobular, interlobular connective»adipose
No. of ductules/TDLU (Types 1, 2, and 3, respectively)	N/A	11, 47, 81	9, 24, 64	Not defined
No. of galactophores	1	~8–15	2	1
Epithelial proliferation	Concentrated in end buds and alveoli	Concentrated in end buds or TDLU	Concentrated in end buds or TDLU	Peripheral zones of TDLU
Response to estrogen	End buds	Epithelial	End buds, TDLU	Epithelial
Response to progesterone	Alveoli	Negative, or no effect	No effect	No effect
Mammary tumors	Spontaneous, viral origin	Spontaneous	Rare, few cases	Rare, few cases
Tumor precursor	AH, ADH, DCIS	ADH, DCIS	Unknown	Unknown
Tumor hormone dependence	Rare	50%–60%	Unknown	Unknown

ADH, atypical ductal hyperplasia; *AH*, alveolar hyperplasia; *DCIS*, ductal carcinoma in situ; *TDLU*, terminal ductal lobular unit.

can be selected according to the biological aspect of interest.

In order to distinguish tissue test systems from in vivo human or animal models and refine the expectations of an in vitro model, a tissue test system can be defined as a modular unit of "useful" biology. A tissue test system is depicted in Fig. 30.2 as a tissue model built using a Lego-like approach with biological and nonbiological components that can then be used to answer biological questions. This Lego-like approach may be extended to interconnect complementary tissue-engineered organ modules to model more complex phenomena [122,123]; Clemson University Research Foundation (disclosure 2005-32) such as drug pathways and pharmaceutical tissue interactions. The three primary questions that must be considered in designing a test system are as follows:

1. What is the biological question of interest and how can this question be answered with an approximate biological model?
2. What are the elemental components (i.e., the Lego blocks) and how are the elemental components arranged to make the biological model?
3. How will the elemental components be assembled and the final tissue cultured?

One cannot survive in the world without mentally forming cause and effect relationship models that provide useful advice about behaviors of the systems around us. Many natural systems are understood through models, which provide useful information, such as predicting the future state of a system for a change in inputs to the system. As an example from physics, the behavior of a resistance may be described by the simple Ohm's law mathematical model of the voltage across the resistance equals the resistance multiplied by the current, $v = Ri$. This model is "useful" to make calculations such as how many light bulbs could be connected to an electric circuit. The Ohm's law model is easy to use, scales to more complex problems, is consistent and reliable since it works for anything that behaves like a pure resistance over a specific operating range, has been verified by experimentation, is cost effective to use, can be extended to account for additional physical phenomena, and increases understanding of the system. Thus even though the Ohm's law model is not a copy of the resistance, it provides useful information. A tissue test system holds the same promise to provide a model that facilitates the creation and sharing of new knowledge of breast tissue processes.

The Ohm's law resistance model has obvious limitations. When the model is tested under unmodeled conditions such as very high temperatures, the information from the resistor model becomes less accurate and hence less "useful." In this case the resistance model can be updated by changing the constant resistance to a function

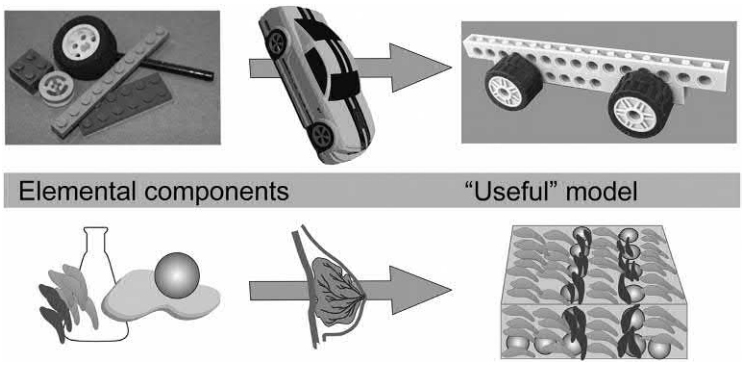

FIGURE 30.2 Top row shows the Lego approach where plastic blocks are used to build a model of a car. This model is simple, representing one specific aspect of the car; however, the complexity of the model can be increased to match the end goal, that is, to isolate the element(s) of interest. Clearly models cannot, and are not intended to, replicate all aspects of a functional system and are only valid within their stated, limited bounds. The analogy to biofabrication of a tissue test system is shown in the bottom row where the elemental components, for example, cellular units, biomaterials such as beads, fibers, or gels, and biochemical agents are assembled to produce relevant aspects of a tissue, that is, a tissue model.

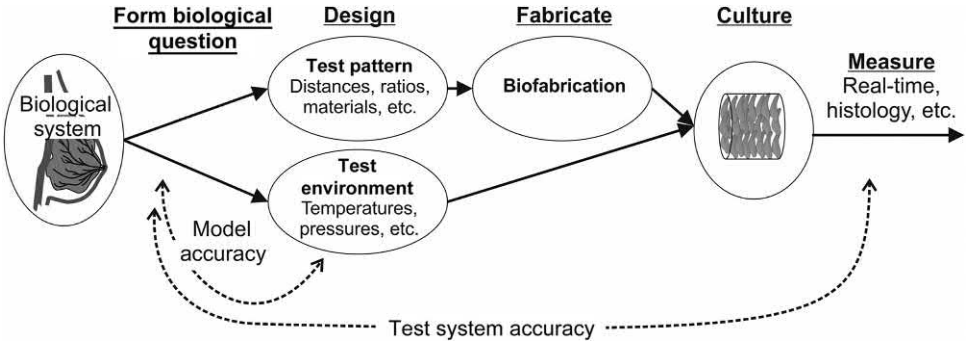

FIGURE 30.3 Process of developing biologically relevant tissue test systems. A final relevancy/benchmarking check is conducted to determine if the test environment is providing meaningful information, relevant to the biological system (again realizing that a model approximates and simplifies, it does not replicate).

of temperature, that is, $R(T)$. Specifically, the resistor model must be designed to include the phenomena that affect the accuracy and resolution needed to provide useful information for a specific question of interest. Thus a breast tissue test system must be defined to match the expectations of the user to provide useful information. Practically, a tissue test system must be defined to meet the user's needs and should not be a quest to reproduce the full in vivo biology; rather, it should be a design approach to capture only the salient physiological, mechanical, biochemical, morphological, and biological elements needed to study a specific phenomenon.

A general schematic of the process of designing, fabricating, and using a tissue test system is shown in Fig. 30.3. Tissue test system development begins with assessing the physical system to identify the scope and features needed in the test system model. The first challenge in using a tissue test system is defining what behaviors in the native system must be included to address a specific question of interest; that is, what does one intend to learn from the tissue test system and what are the first-order factors that affect the behavior under study. Model accuracy is used here to describe the translation of a biological question into a test pattern or structure for the tissue test system. Note that high model accuracy does not mean that the test pattern must be an exact reproduction of the biological structure, in this case the breast; rather, it must be a representation of the underlying chemical, physical, electrical, etc. phenomena that are useful for answering a specific biological question regarding breast tissue. In fact, a well-designed test system will exclude factors that confound answering the biological question of interest. Hence, different biological questions will require different test system models. As an example, there are currently test systems, such as those addressing adhesion assays, that comprise cells seeded onto a plate. These are relatively straightforward test systems to implement and hence are often the first assays performed. Such a flat, 2D system can answer important questions; for example, adhesion assays were used to study the expression and function of Laminin-511 during metastatic breast cancer progression [124]. These results can inform decisions regarding incorporation of Laminin-511 in a breast tissue implant, or regarding selection of reconstructive biomaterials that may promote or inhibit production of this extracellular protein. Note, however, that 2D cultures are not amenable to the study of breast ductal structures or questions regarding spatial colocation [125]. Following Fig. 30.3, the test pattern must be fabricated, for example by biofabrication, and then cultured. The biofabrication system to produce a desired test system and then the culture system, for example, a bioreactor, to produce the environmental conditions, such as temperature or pH, present significant instrumentation challenges. Test system accuracy describes the overall degree to which the biological question of interest can be studied in the in vitro culture.

An established tool in breast cancer studies that is also applicable to breast reconstruction research is the breast

tissue organoid, which is formed by digesting harvested tissues into duct-like structures that resemble the original organ in appearance or function. While it is obvious that an organoid model formed by removing tissue and allowing the remaining tissue to grow in a new manner is not a physical copy of the breast tissue, it has been shown that this simplified model can produce useful results. For example, in the work by Cellurale and coworkers [126] the authors were able to isolate the role of cJun NH2-terminal kinase signaling in mammary gland development and tumorigenesis using mammary organoid cultures. In other studies [127], organoids were used as the basic building block of human mammary epithelial cell cultures. The organoid model uses an elemental component that already has significant structure and is directly derived from the biological source. Researchers developed a protocol and created over a hundred breast cancer organoid lines for research and drug development [128].

There are more general approaches to building test systems that use biofabrication techniques to assemble smaller elemental pieces. Biofabrication is the process in which cells, biomaterials, and biochemical agents are arranged into a biological structure. The components of materials must be chosen and assembled such that they promote cellular growth and behaviors. The specific fabrication technologies dictate the degree to which the materials and cells can be arranged in heterogenous patterns. Current discrete deposition techniques can be labeled as "quantum-on-demand" bioprinting as small units of cells or biomaterials are used to build the biological structure. Fig. 30.4 illustrates three general modes for depositing a quantum of cells and biomaterials at a specific time or location [123]. The "cells-on-demand" mode describes the direct placement of cells in the structure. The "liquid/gel-on-demand" mode describes placing an extrudate, a cellular or acellular "bioink," at precise locations. The "particle/bead-on-demand" mode involves the deposition of a hard biomaterial particle or bead. Fig. 30.5 shows considerations in biofabricating a test system model. The first source of potential error is the discretization of a proposed test pattern based on the resolution of the fabrication system. The 2D pixel error is illustrated in the second column of Fig. 30.5 where it can be seen that the biofabrication system with the coarser resolution produces a lower fidelity replica of the test pattern. The 3D equivalent of pixel error is the voxel error, which includes the pixel error as well as a depth component. For example, if a test pattern is defined at the cellular level using micron resolution, such as a scan of a histology slide, then a biofabrication technique that has the smallest

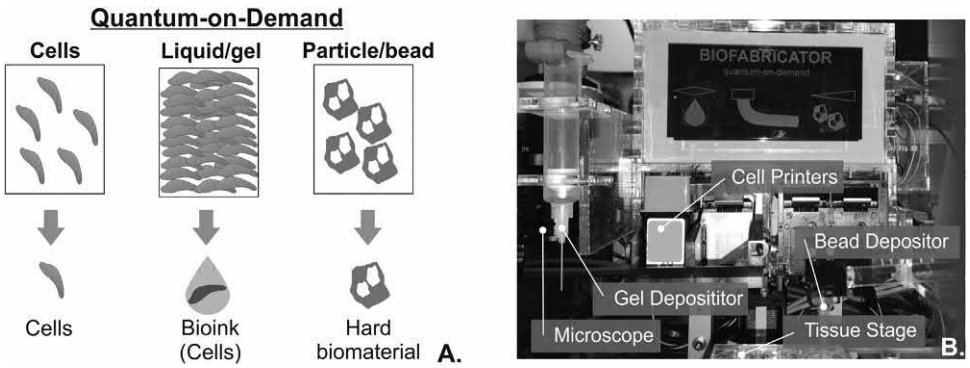

FIGURE 30.4 (A) The three general modes to apply a quantum of cells and/or biomaterials during biofabrication of tissues and (B) photo of a biofabrication system with multiple deposition stations for cells and biomaterials.

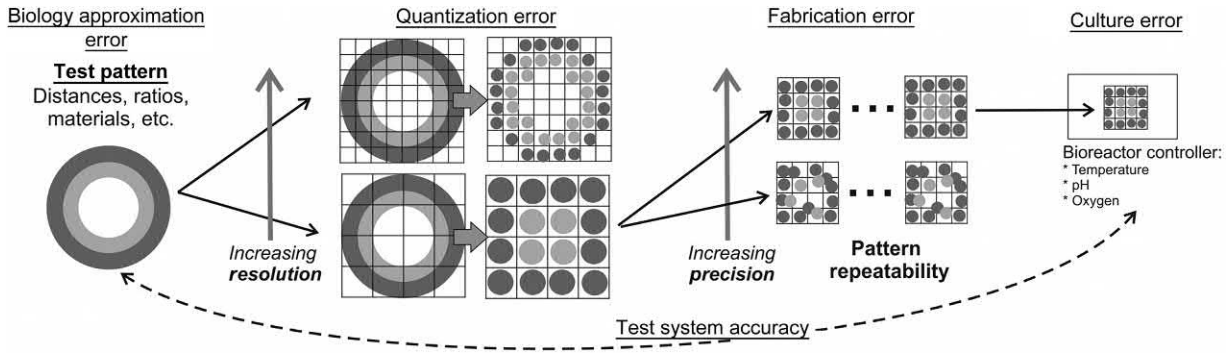

FIGURE 30.5 Sources of error in biofabrication of tissue test systems include the quantization error that arises from resolution of the fabrication instrumentation, the fabrication error that arises from the precision and repeatability of the fabrication instrumentation, and the culture error that arises from the ability of the culture controller to reproduce environmental conditions.

fabrication component in the order of a millimeter can only produce an approximate copy of the desired pattern. Thus the approximation of continuous biology with discrete fabrication components leads to quantization error.

The second fabrication error type is in the ability of the biofabrication technique to result in the placement of elemental components at a desired location. As illustrated in the third column of Fig. 30.5, high precision means that the system can closely replicate the discretized test pattern. The ability of a process to produce identical replicates of a test pattern is then defined as the pattern repeatability. This is defined as different from the machine precision because occurrences such as cell settling in storage reservoirs can cause evolution of the biofabrication instrumentation precision over time. Note that there is much debate about how much quantization error and fabrication error can be tolerated in building a useful tissue structure. One consideration is that tissue structures will self-assemble from an appropriate starting condition [129]. Specifically, placing cells "close enough" will allow them to move and assemble based on their natural behaviors. Any approach to tissue fabrication will require that nature take over for the final stage of tissue assembly, the idea of "close enough" is actively debated.

The ability of the tissue culture system to reproduce environmental conditions can greatly affect overall results, the culture error is shown in the last stage of Fig. 30.5. An important part of specifying the tissue test system is to define the environmental conditions, for example, dissolved oxygen level, that will be used to incubate the tissue. The capability of the culture system to produce these conditions must be considered; limitations occur in the ability of the instrumentation to measure a quantity of interest and the ability of the control system to maintain or modulate that quantity. As with all aspects of the tissue test system, the environment is a simplified model of the actual environment and as such must be designed at the start of a project to address a specific question of interest.

As a final consideration, the state or feature of interest must be measured in the tissue or medium during and/or after culture. A tissue test system can present the same imaging and measurement challenges as in vivo experiments—real-time measurements such as oxygen levels, discrete measurements such as magnetic resonance imaging, and endpoint analysis such as classical histology may all be used but have the same fundamental instrumentation limitations. However, there are several important advantages of a tissue test system over an in vivo model. First, there is the possibility that sensors can be embedded directly into the tissue design. For example, it is possible to embed a temperature sensor in the center of a tumor mass as it is fabricated. Second, the tissue is potentially more amenable to direct observation than in vivo, for example, a tumor model could be grown on a microscope stage and images automatically taken at regular intervals to observe spreading or growth. There is generally improved access to both inputs and outputs of the culture in a test system, agents can be applied without metabolism or complex pharmokinetics distorting the input. As an example, it is possible to apply an anti-angiogenic agent to a cell culture and know the local concentration without measurement because the pharmokinetics can be easily modeled as a mixing problem in a fixed volume. Similar arguments suggest that observation of outputs, such as metabolic byproducts, are more accessible in a test system. Perhaps the most important advantage that the tissue test system can have over an in vivo test is that, given low fabrication errors and culture errors, each copy of the test tissue should be nearly identical. This means that replicates can increase the power of observations and increase the ability of outside groups to repeat experiments and results. In general, a tissue test system should make the biology of interest more accessible for observation and manipulation.

In summary, tissue test systems are an evolving approach to modeling complex biological systems in vitro. Ethical, economic, and scientific drivers will ensure that this technology continues to evolve. However, the use and expectations of the test system must recognize that the tissue and culture environment is an approximate model that must be designed to address a specific question of interest. As researchers and clinicians begin to appreciate all of the subtleties of designing, fabricating, culturing, and assessing tissue test systems, these approximate models will help reveal the complexities of breast biology and disease processes. As tissue test systems, the basic units of useful biology, become standardized, they can be integrated to produce systems capable of answering ever more complex questions.

In silico breast cancer models

Mathematical modeling of breast cancer continues to advance rapidly, so much so that any summary is nearly obsolete at the time of writing. However, a brief overview of some approaches helps demonstrate the potential and challenges of mathematical and computer models. Generally speaking, there are two starting points for deriving a mathematical model, the first is the application of basic chemical and physical equations (first principles) to create a model and the second is the observation of physical behavior and use of mathematical tools to capture this behavior. A model may be proposed to work at a specific scale, such as at the molecular level, the cellular level, the organ level, the organism-wide level, or the population level. Initially, models were developed to

capture the behavior of an isolated system at a fixed scale. As modeling sophistication has increased, multiscale models [130] of connected subsystems have evolved. Often the models are referred to as "in silico" to indicate that the simulation is performed on a computer and to highlight their similarity in role to in vivo or in vitro models. As with other modeling approaches, the best model is the one that allows the user to address the question at hand.

As an example of a simple model, tumor angiogenesis is modeled as a set of two interconnected differential equations, one subsystem models tumor growth and the other subsystem models the carrying capacity of the vascular network [131]. This model appears to be a gross summary (as an ordinary differential equation) of the physical diffusion processes and the effects of the biological signals that stimulate or inhibit angiogenesis. However, analysis of the model has provided new insight into the scheduling of antiangiogenic treatments. The traditional dosing of such a therapy centers on applying a constant dose at regular time intervals; however, the model provides the structure to apply optimization and control techniques that suggest a more efficient use of the treatment agent [131,132]. There will always be questions about the resolution and accuracy of any mathematical model. For example, the angiogenesis model may not be sufficient to model clinical cancer treatment. By connecting a second subsystem model, the movement of bone marrow–derived endothelial progenitor cells to the tumor site and their effect on tumor growth can be used as a starting point to model vasculogenesis [133]. Such a model can clarify further the possible treatment strategies, including chemotherapy and anti-angiogenic therapies, aimed at suppressing vascularization, which may be incorporated in a tissue-engineered implant.

Concluding remarks

Tissue engineering may provide a means of both assessing and building reconstructive tissue implants for a woman undergoing lumpectomy or mastectomy. The development of vascularization and the long-term retention of tissue volume are keys to viable breast reconstruction options. Benchtop tissue test systems will be critically important in addressing these two major issues, by facilitating markedly improved understanding of the normal and diseased states and, accordingly, assessing and developing advanced reconstructive options.

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Part Nine

Cardiovascular system

Chapter 31

Cardiac progenitor cells, tissue homeostasis, and regeneration

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Origin of cardiac stem/progenitor cells

Adult cardiac stem/progenitor cells are thought to be derived from the cells that form the heart and related structures during development. The heart is derived from four distinct cardiac precursor cell lineages, the first and second heart fields (FHF and SHF, respectively), the proepicardial organ (PEO), and the cardiac neural crest (CNC). Each of these cardiac precursor cell lineages forms at different stages during embryogenesis. Initially, a continuum of epithelial-to-mesenchymal transitions (EMTs) in the epiblast lead to the gastrulation of the FHF, SHF, and PEO cardiac precursors as parts of the mesodermal germ layer [1,2]. The CNC will arise later, at the stage of neurulation, in response to transient EMT at the junction of the neural plate and nonneural ectoderm.

Upon gastrulation the FHF precursors migrate cranially and coalesce into a crescent-like shape—the cardiac crescent—which will morph into the linear heart tube. These cells will proliferate and differentiate into the majority of the left ventricular free wall, as well as regions of the interventricular septum (IVS) and atrial myocytes and vessels. The SHF, added medially to the cardiac crescent, is patterned into an anterior and posterior segment. The anterior SHF gives rise to the right ventricle, portions of the IVS, and part of the outflow tract (OFT); while the posterior SHF produces the remaining parts of the atria, parts of the OFT and is also thought to contain precursors of cardiac conduction system myocytes [3−6]. The PEO, a cauliflower-like structure that develops at the inflow region of the looping heart tube [7−9], produces the epicardium, myofibroblasts and contributes to the development of the coronary vasculature [10−13]. The CNC contributes to cardiac and aorticopulmonary septation, valvulogenesis, and the autonomic nerves [14].

Each of these four lineages is specified through the induction of specific genes and signaling pathways during the development, a process that is influenced by the local concentration of growth factors and other morphogens (see Refs. [15,16]). Whether any of these embryonic cardiac precursor cell programs is transmitted into the postnatal life and persist as resident adult cardiac stem/progenitor cells, remains unclear.

The FHF and SHF lineages are specified before gastrulation, with the FHF and SHF derived from an early and late wave of cells, respectively, expressing the mesoderm posterior 1 (*Mesp1*) gene. A crucial event in cardiogenesis is the response of these cells to the morphogen wingless-type MMTV integration site (Wnt) family member proteins. Canonical Wnt (e.g., Wnt3a) signaling activates ß-catenin, a protein that has myriad effects, both as a transcription factor and extranuclear. While Wnt signaling early in development induces the mesoderm, later exposure to Wnt inhibits cardiogenesis posterior to the cardiac crescent while promoting cardiomyocyte differentiation of FHF and SHF cells. These effects are mediated, in part via simultaneous exposure of these cells to other factors/morphogens such as bone morphogenetic protein (BMP)-2 and -4, fibroblast growth factors (FGFs) and others (see Ref. [15] for review).

Following differentiation, cardiomyocytes proliferate extensively, a process controlled, at least in large part, by the Hippo/Yes-associated protein (Yap) pathway. Hippo/Yap helps regulate the growth of many organs during the development. Overexpressing a constitutively active YAP in cardiomyocytes stimulated proliferation [17,18]. Yap

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appears to interact directly with ß-catenin to control cardiomyocyte proliferation and heart size [19].

The FHF and SHF have common and distinct gene expression profiles. Both progenitor populations express transcription factors crucial for cardiomyocyte differentiation: the homeodomain transcription factor NK2 transcription related, locus 5 (Nkx2-5), and GATA binding protein 4 (Gata4). However, FHF cells express T-box transcription factor 5 (*Tbx5*) and the basic helix-loop-helix transcription factor *Hand1*, whereas SHF progenitors express *Hand2*, Islet1 (*Isl1*; which may also be expressed in the FHF), forkhead box h1 (*Foxh1*), FGF 10 (*Fgf10*), and *Mef2c* [3,20,21].

The PEO is derived from an early wave of $Mesp1^+$ progenitors, which are thought to be distinct from the FHF/SHF-forming $Mesp1^+$ cardioblasts [22]. These progenitors ultimately give rise to the epicardium, a layer of cells that delineates the outer layer of the primordial heart and contributes to the development of the coronary vasculature and myofibroblasts, although not cardiomyocytes. However, the epicardium does promote cardiomyocyte proliferation during development via its secretion of growth factors such as insulin-like growth factor 2 [23], FGF, and (perhaps) Follistatin-like 1 [24] (Fig. 31.1).

Early during heart development, proepicardial cells undergo EMT and attach to the myocardial surface, where they proliferate and migrate to form a continuous cell layer. In humans the atrial epicardium is a monolayer as is the entire epicardium in other organisms [26]. The migrating epicardial derived progenitor cells (EDPC) differentiate into perivascular and interstitial fibroblasts. Murine EDPCs can also differentiate into vascular smooth muscle cells, pericytes, and, as mentioned previously, mesenchymal stem cell (MSC)-like cells (CMSCLCs). However, their ability to differentiate into cardiomyocytes appears to be very limited; and despite initial reports, EDPCs are likely not a source of endothelial cells (ECs). Zebrafish and mouse models illustrate that perturbing the differentiation of EDPCs produces abnormalities in cardiac structure [27].

EDPCs are clearly not a homogenous population. The cells express one or more of a number of transcription factors, including Sema3d, Scx, Tbx18, Tcf21, Raldh2, and Wt1, which can affect the differentiation potential of EDPCs (see Ref. [28] for review). While characteristic, these markers are not exclusively seen in EDPCs. The epicardium of the healthy adult heart is normally quiescent but can be quickly activated following injury.

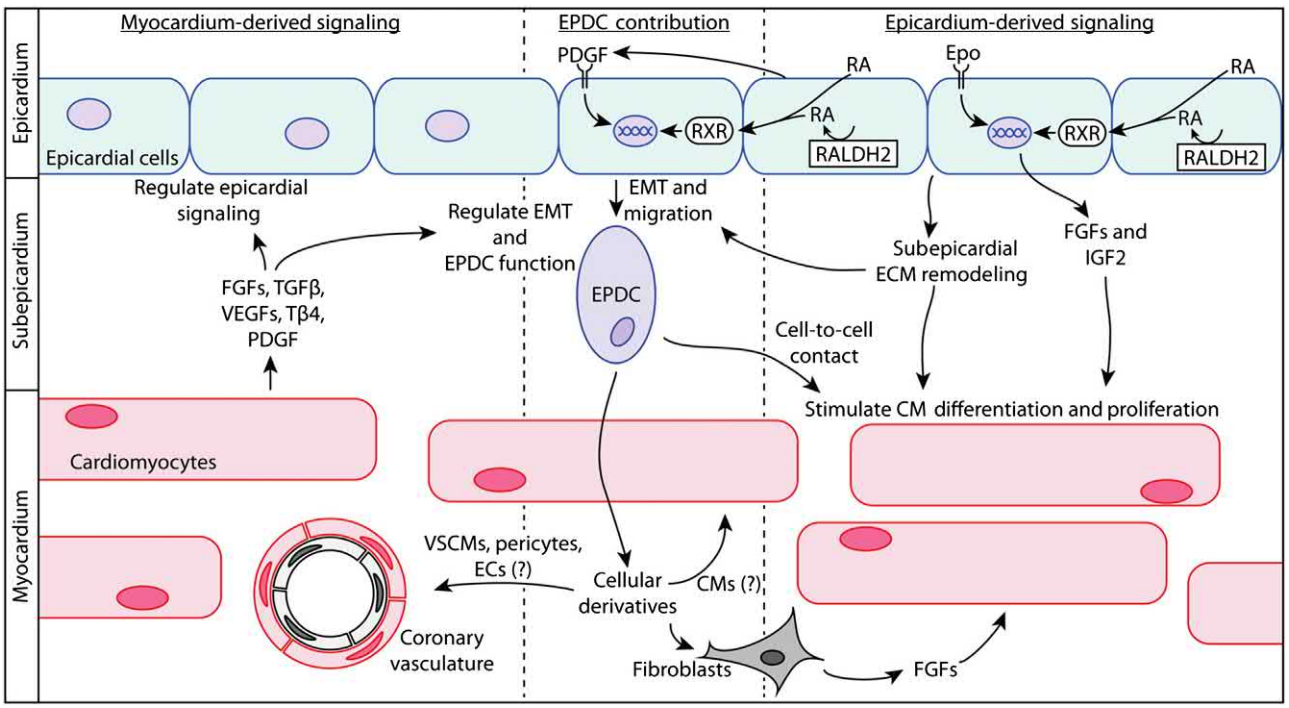

FIGURE 31.1 Epicardial cell contribution and reciprocal epicardial–myocardial signaling are critical for cardiac development and may similarly determine epicardial potential for cardiac regeneration. *CM*, Cardiomyocyte; *EC*, endothelial cell; *EMT*, epithelial-to-mesenchymal transition; *EPDC*, epicardium-derived cell; *Epo*, erythropoietin; *FGF*, fibroblast growth factor; *IGF2*, insulin-like growth factor 2; *PDGF*, platelet-derived growth factor; *RA*, retinoic acid; *RALDH2*, retinaldehyde dehydrogenase 2; *RXR*, retinoid X receptor; *T4*, thymosin 4; *TGF*, transforming growth factor; *VEGF*, vascular endothelial growth factor; *VSMCs*, vascular smooth muscle cells. *From Duffey OJ, Smart N. Approaches to augment vascularisation and regeneration of the adult heart via the reactivated epicardium. Glob Cardiol Sci Pract 2016;2016(4):e201628.*

EDPCs revert to a gene expression pattern reminiscent of fetal cardiac development. Following myocardial infarction (MI), *Wt1*, *Raldh2*, and *Tbx18* are upregulated in what appears to be a process controlled by the C/EBP family of transcription factors [29] and BRG1, an ATPase subunit of the SWI/SNF chromatin-remodeling complex [30]. This reactivation of developmental gene expression patterns is also induced by thymosin ß4, an actin sequestering protein that interacts with BRG1. However, the thymosin ß4 effects are seen only when it is administered prior to MI [30]. The upregulation of Wt1 likely promotes migration of the newly (re-)activated epicardium resulting in the rapid replacement and thickening of the epicardium lost at the injury site. During this repair process the activated epicardium also appears to stimulate cardiomyocyte proliferation (Fig. 31.1), reminiscent of its function during development (see Refs. [25,28,31] for review).

CNCs are $Mesp1^{-}$ cells and are derived from the neuroectoderm [32]. Neural crest cells are a crucial component of mammalian development and differentiate into a wide variety of structures ranging from the bones, cartilage, and muscles of the head and neck, to melanocytes and parasympathetic ganglia. In the heart the CNC contributes to cardiac and aorticopulmonary septation, valves, and the autonomic nervous system [14]. Approximately 10% of cardiac myocytes in mice and fish appear to be derived from the CNC [16,33–35]. Hatzistergos et al. demonstrated that CNCs expressing c-kit [CD117, the receptor for stem cell factor (SCF) [36]], become cardiomyocytes in the atria, ventricles, and IVS as well as pericardial, endocardial, and epicardial cells [16]. Adult cardiac progenitors may be the descendants of these CNC. The number of different stem/progenitor cell populations that reside in the adult heart, their role in maintaining homeostasis in the healthy heart, the extent of their participation in repairing the damaged heart, their ability to differentiate into cardiomyocytes, EC, and/or vascular smooth muscle is controversial and is being actively studied.

Modeling cardiac development with pluripotent stem cells

The ability to model cardiogenesis using pluripotent stem cells (PSCs), that is, embryonic stem cells (ESCs) and induced PSCs (iPSCs), has greatly expanded our understanding of the origin and differentiation capabilities of cardiac progenitors. ESCs are pluripotent cells derived from the inner cell mass of mammalian late blastocyst embryos. iPSCs are adult somatic cells that have been reprogrammed into pluripotent cells through the introduction of a combination of specific factors [37,38]. PSCs can be differentiated into cardiomyocytes by following well-established protocols involving the transient inhibition and subsequent stimulation of Wnt/ß-catenin signaling [39,40].

Human (h) ESCs (hESC) and iPSCs that express the signal-regulatory protein α produced a population of cardiac precursors and cardiomyocytes that were virtually all cardiac troponin T + and could contract. Other cell surface proteins, such as PECAM, THY1, PDGFRß, and ITGA1, identified nonmyocyte cells [41]. Wu et al. [42], Christoforou et al. [43], and Hatzistergos et al. [16] showed that a fraction of mouse PSC-derived $Nkx2\text{-}5^{+}$ cardiomyogenic cells express c-kit on the cell surface.

Another advantage of using PSCs is their ability to differentiate into cardiomyocytes with atrial or ventricular characteristics. For example, Lee et al. showed that ventricular and atrial cardiomyocytes are derived from different mesoderm populations. Ventricular cardiomyocyte progenitors express Glycophorin A (CD235a), whereas atrial cardiomyocyte progenitors express retinaldehyde dehydrogenase 2 (RALDH2) [44]. This study as well as a more recent study by Lemme et al. [45] demonstrated that retinoic acid is a critical factor for specifying human atrial cardiomyocytes.

Hatzistergos et al. used a murine PSCs model of CNC development to demonstrate that the differentiation of $c\text{-}kit^{+}$ cardiac progenitors into cardiomyocytes is regulated by BMP. They observed that in the presence of BMP, few cardiomyocytes were produced, but exposing the cells to a BMP antagonist (e.g., dorsomorphin) promoted the differentiation of these neural crest cells into beating cardiomyocytes [16].

Exposure of hPSCs to BMP4 from early stages of cell culture results in cells that express *WT1* with or without expression of *Tbx18*, both epicardial markers [10]. Treatment with the BMP antagonist, Noggin, abrogated the differentiation into epicardial cells. In addition to BMP4, epicardial cell differentiation also requires Wnt signaling. Therefore formation of epicardial cells requires signals opposite to those required for cardiomyocyte formation [10]. Furthermore, these epicardial cells undergo EMT following exposure to transforming growth factor (TGF)-ß and basic FGF (bFGF) [10] (Fig. 31.2). These results suggest that the local environment into which progenitor cells migrate or reside during development is a key factor regulating their differentiation capacity. Studies in hPSCs also demonstrate that stage-specific activation of tumor suppressors (negative cell cycle regulators) promotes the cardiomyocyte development [46].

Zhang et al. recently used a dual reporter system to isolate and examine hiPSCs that differentiated into four lineages; FHF, SHF, epicardium, and endothelium based on positive or negative expression of *Tbx5* and *Nkx2-5*. Using this approach, they identified cells with characteristics of ventricular or atrial myocytes, the sinoatrial node

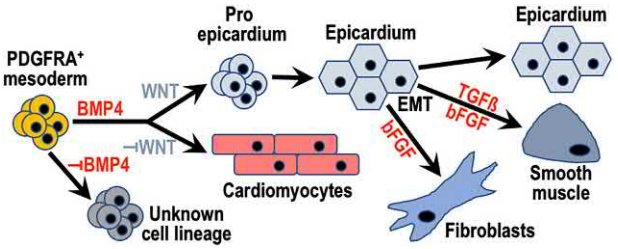

FIGURE 31.2 Model highlighting the specification of PDGFRA + mesoderm to the cardiomyocyte and proepicardial lineages by BMP4 and canonical Wnt signaling. When passaged in the absence of factors, the proepicardial cells form epithelial sheets resembling the epicardium that can be identified through the aldefluor assay. In the presence of TGF-ß + bFGF or bFGF, the passaged cells undergo EMT and give rise to smooth muscle-like and fibroblast-like cells, respectively. *bFGF*, Basic fibroblast growth factors; *BMP*, bone morphogenetic protein; *EMT*, epithelial-to-mesenchymal transition; *TGF*, transforming growth factor. *Modified from Witty AD, et al. Generation of the epicardial lineage from human pluripotent stem cells. Nat Biotechnol 2014;32 (10):1026–35.*

(SAN), or endothelium. They then assessed each lineage for drug responsiveness and concluded that this methodology has potential clinical value [47]. Other studies using hPSCs have modeled the formation of the SAN [48] and cardiac valves [49].

Analyzing the transcriptome [50] and chromatin structure/accessibility of human PSC subpopulations [51] has identified important regulatory pathways associated with cardiac progenitor differentiation. Techniques, such as CRISPR/Cas9 and novel inhibitors/stimulators of signaling pathways, have increased our ability to manipulate pluripotent cells and will expand our understanding of the development, differentiation, and therapeutic potential of cardiac progenitors in a manner not previously possible.

In vivo fate mapping of cardiac progenitors

Identifying the existence, progeny, and differentiation ability of cardiac progenitors is possible in vivo using genetic fate-mapping techniques. In this approach, cells are conditionally labeled, often beginning at specific stages of gestation, and the pre- and/or postnatal expression of the marker is then assessed. Most of these studies use mice with two genetic manipulations, a label that is normally inactive in all cells and a transgene comprised of a recombinase (e.g., Cre) controlled by a specific (truncated) promoter. When the promoter is activated, expression of the recombinase occurs in a cell- or tissue-specific manner. The recombinase acts on the DNA to activate expression of the label only in those cells. All cells derived from the labeled cells are similarly labeled. Expression of the recombinase can be further controlled temporally (e.g., by tamoxifen administration) so that the label can be activated in the tissue beginning at a specific stage of gestation or postnatal life. Genetic fate mapping can also be done in zebrafish (e.g., Ref. [52]). These fate-mapping approaches can produce ectopic expression of the label resulting in conflicting/confusing results; but improvements to the system are ongoing (e.g., Ref. [53]).

In vivo fate mapping has been used to assess the source(s) and differentiation ability of c-kit$^+$ cardiac cells. The role of c-kit$^+$ cells in cardiomyocyte development and regeneration is controversial [54]. These cells were originally thought to comprise multipotent cardiogenic mesoderm-derived postnatal myocardial progenitors that persist in the postnatal heart [42,43,55]. However, lineage-tracing studies of cardiac c-kit$^+$ cells using an intersectional genetic fate-mapping approach showed that expression of c-kit is rarely activated in the myocardial lineage and marks CNC rather than mesoderm-derived myocardial cells [56]. Furthermore, genetic fate-mapping studies in mice indicate that expression of c-kit in the cardiac mesoderm is largely associated with coronary endothelium rather than myocardium-producing cells [56] (Fig. 31.3).

A similar intersectional genetic fate-mapping approach was recently devised by Li et al. to address the role of resident cardiac progenitor/stem cells in postnatal cardiomyogenesis [59]. This study examined the ability of nonmyocytes (including putative cardiac progenitors) to convert into cardiomyocytes in embryonic and in healthy and injured adult mouse hearts. Using a newer gene-mapping technique [53], they found evidence for nonmyocyte to myocyte conversion in embryonic hearts but not in adult hearts. Their results did not provide evidence for the existence of cardiac stem cells (CSCs) in the adult during homeostasis or after injury [59]. However, these mapping studies provide only indirect measures of activity/lineage tracing and likely do not measure cells that are inactive or silenced.

An alternative approach to understanding the ontogeny and progression of cardiac progenitors is through single cell RNA sequencing. Cells are isolated at specific stages of cardiac development and a cell-specific transcriptome established that enables the identification of distinct populations of cardiac progenitors. For example, Lescroart et al. identified subsets of Mesp1$^+$ cardiac progenitors that become committed to different cell lineages during the heart development. This approach represents a first step toward characterizing the molecular signature associated with this stage of lineage restriction [60].

Neonatal cardiac repair

Unlike the adult heart, the neonatal mammalian heart is capable of completing regeneration if the injury occurs in the first few postnatal days (P). Partial apical resection or

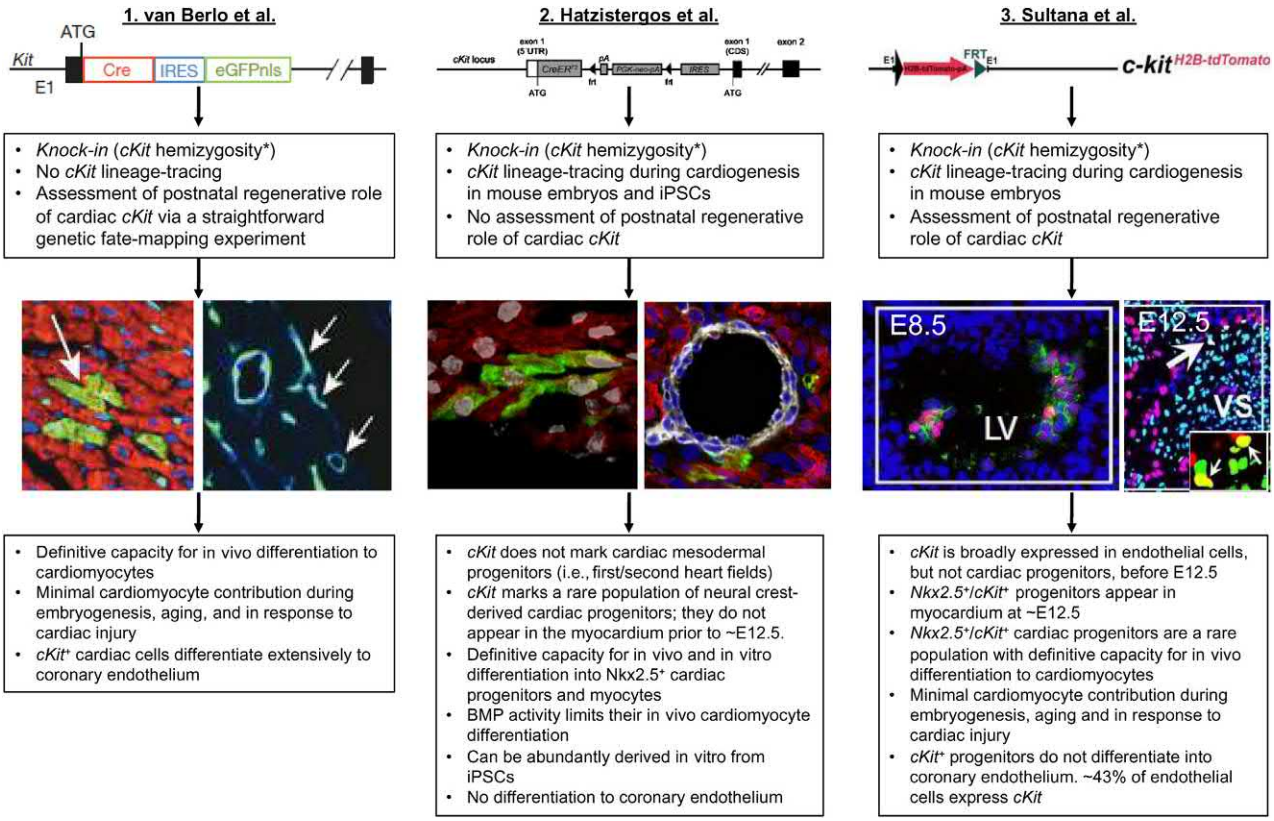

FIGURE 31.3 Differing opinions on the extent of myocardial contribution of c-kit$^+$ cells. c-kit$^+$/Nkx2.5$^-$ vasculogenic cells and c-kit$^+$/Nkx2.5$^+$ cardiomyogenic lineage are derived from cardiac neural crest cells. These three genetic lineage fate-mapping studies demonstrate that cardiac c-kit$^+$ cells form cardiomyocytes in the murine heart at a low level but differ on endothelial differentiation. van Berlo et al. [57] showed that c-kit$^+$ cardiac cells exhibit extensive endothelial cell differentiation. Hatzistergos et al. [36] and Sultana et al [58] disagree, although the latter shows that ≈43% of coronary endothelial cells are c-kit$^+$ (possibly at low levels). Hatzistergos et al. [36] and Sultana et al. [58] both establish that c-kit$^+$/Nkx2.5$^+$ cardiomyogenic progenitors are only present after embryonic day (E)12.5. *Note*: All three studies used knockin mouse models in which the gene-targeted c-kit allele was nonfunctional, an approach that may produce abnormalities in c-kit$^+$ cardiac cells in these mice (e.g., proliferation, migration, and differentiation). *BMP*, Bone morphogenetic protein; *LV*, left ventricle; *VS*, ventricular septum. *Reproduced with permission from Hatzistergos KE, Hare JM. Murine models demonstrate distinct vasculogenic and cardiomyogenic cKit + lineages in the Heart. Circ Res 2016;118(3):382–7.*

ischemic MI performed on neonatal mice at P1–2 was completely healed within 21 days; however, incomplete regeneration occurs following cryoinjury or if the resection is too large (see Ref. [61] for review). The capacity for complete cardiac repair rapidly declines after the first two days of life and by P7, regenerative capabilities are similar to the adult, leading to incomplete recovery and scar formation [61,62]. Proliferation of resident cardiomyocytes is the underlying mechanism of repair [62] and is promoted by activation of the Hippo/YAP pathway [18,63] but inhibited by the microRNA (miR)-15 family [64]. Other factors appear crucial for neonatal regeneration, including an intact cardiac parasympathetic [65] and sympathetic [66] nervous system (Fig. 31.4).

A recent study demonstrated that MI during P1–2 induces the formation of collateral arteries, allowing for complete cardiac repair [68]. This novel mechanism, termed "artery reassembly," involves arterial ECs migrating to the injury site along capillaries and reassembling into collateral arteries. The arterial ECs express CXCR4, and following injury, appear to migrate in response to capillary-produced CXCL12 (SCF-1), the ligand of CXCR4. Deletion of CXCL12 or CXCR4 in mice significantly reduced collateral artery formation and regeneration of the neonatal heart [68].

Similar to neonatal mice, P1–2 neonatal pigs can also recover from MI via proliferation of existing cardiomyocytes. The neonatal pigs exhibit minimal fibrosis and normal cardiomyocyte function and number. However, this ability is lost by 3 days after birth [69,70]. Pig and human hearts are very similar in structure and function, suggesting that human neonatal hearts can similarly recover. Neonatal pig hearts highly expressed genes involved in cytokinesis and, like neonatal mice, it appears that this cardiomyocyte proliferation/regenerative program involves an ongoing process at this early stage and is not induced in response to the injury [69,70].

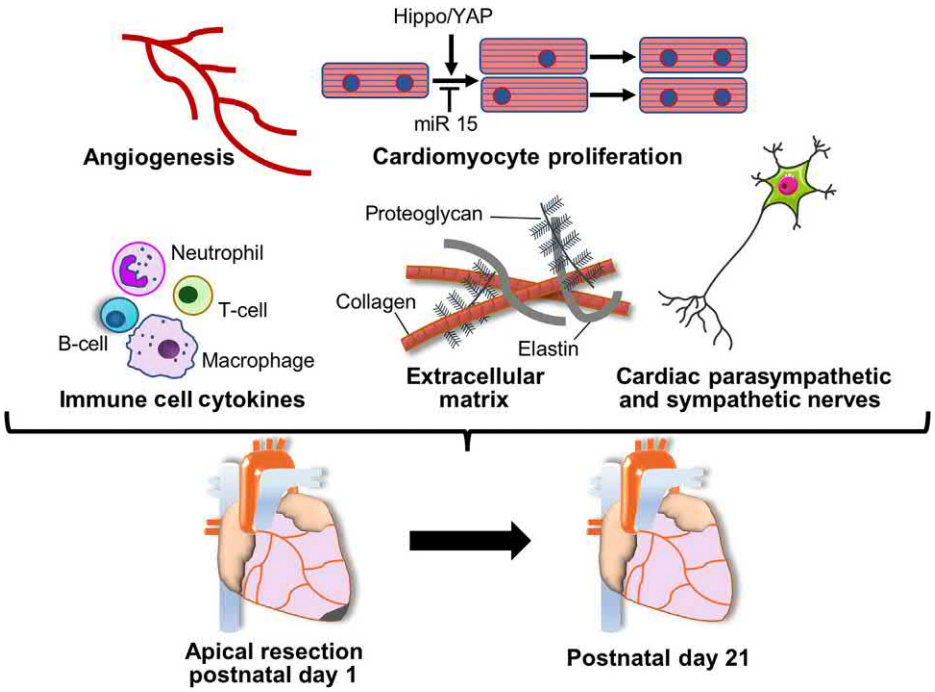

FIGURE 31.4 Factors that promote cardiac regeneration in the neonatal heart. *Adapted from Mahmoud AI, Porrello ER. Upsizing neonatal heart regeneration. Circulation 2018;138 (24):2817–9 [67].*

Reprogramming cardiac fibroblasts

Cardiac fibroblasts are the most abundant cell type in the heart and the possibility of converting them into cardiomyocytes to replace those lost following injury is a very appealing idea. While they are not progenitor cells, per se, cardiac fibroblasts do differentiate into myofibroblasts in response to a variety of stressors (Fig. 31.5) a process that is associated with cardiac injury and progression toward heart failure (reviewed in Ref. [71]). Cardiac fibroblasts can also adopt an osteoblast cell–like phenotype following injury and directly contribute to myocardial calcification. When these fibroblasts are transplanted from an injured to a healthy heart, they promote calcification in the healthy heart. (Fig. 31.5) [72].

Ieda et al. first demonstrated direct reprogramming of cardiac and tail-tip fibroblasts into cardiomyocyte-like cells (induced cardiomyocytes; iCMs) in vitro using viral-mediated overexpression of the cardiomyocyte transcription factors: *Gata4, Mef2c*, and *Tbx5* (a combination they called GMT) [73]. The GMT-transduced cells were injected into NOD/SCID mouse hearts 24 hours later and within 2 weeks, some of these cells expressed α-actinin and exhibited sarcomeric structures [73]. GMT reprograming could occur in vivo and reduced scar size post-MI, an effect that was improved when combined with thymosin ß4 administration [74]. The addition of *Hand2* to GMT (GHMT) or GMT plus *Mesp1* and *Myocd* (GMTMM) more efficiently reprogramed cells (see Refs. [75,76] for review). While there are reports of differentiating human fibroblasts into iCMs in vivo (see Refs. [75,77] for review), this approach is still far from being used clinically.

Cardiac resident mesenchymal stem cells

MSCs are found in all tissues and are identified by their expression of CD105/CD90 and lack of CD45, among other characteristic surface markers, and by their ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages ex vivo [78]. Cardiac resident MSCs (CMSCs)/CMSCLCs are found in adult human [79], mouse [80,81], and swine [81] hearts in proximity to other cardiac progenitors. Monsanto et al. isolated a biopsy from a human heart failure patient, digested it into a single cell suspension and grew the cells overnight [79]. Three cell populations were isolated based on the presence or absence of c-kit. The c-kit$^+$ population was expanded in either cardiac progenitor or endothelial progenitor media resulting in c-kit$^+$ cells that were either CD133 negative or positive, respectively. From the c-kit$^-$ population, CMSCs were isolated based on expression of CD90 and CD105. CMSCs, unlike bone marrow–derived MSCs (BMMSCs), also express cardiac lineage markers such as smooth muscle actin and perhaps GATA-4.

While CMSCLCs may have therapeutic potential, their isolation requires a cardiac biopsy, thereby limiting their potential use. In an attempt to avoid this complication, human CMSCLCs were recently isolated from discarded surgical tissues obtained following coronary artery bypass surgery, primarily from the right atrial appendage [82].

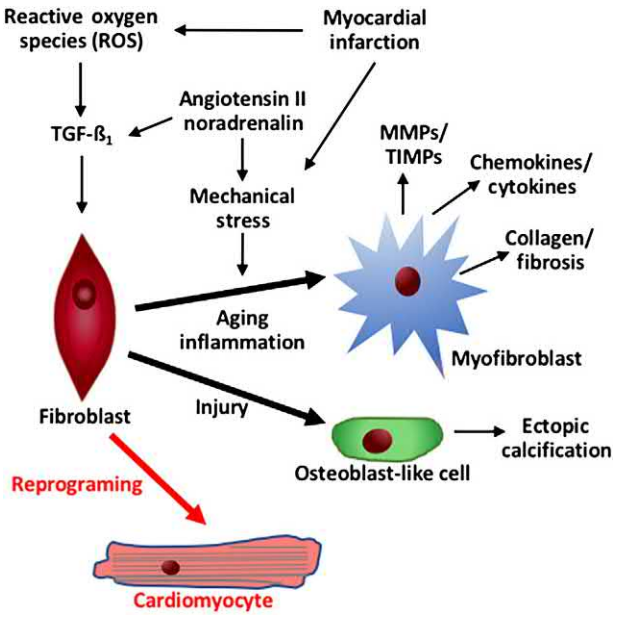

FIGURE 31.5 Fibroblasts can transform into myofibroblasts [71] or osteoblast-like cells in response to a variety of stressors. Myofibroblasts participate in scar formation and osteoblast-like cells produce ectopic calcification of the myocardium [72]. Cardiac fibroblasts can be reprogrammed into cardiomyocytes. *Adapted from Tarbit E, et al. Biomarkers for the identification of cardiac fibroblast and myofibroblast cells. Heart Fail Rev 2019;24(1):1–15.*

These cells possessed some consensus MSC characteristics $CD105^+/CD166^+/CD44^+/MHC\ I^+/MHC\ II^-$ [78]. However, they only rarely differentiated into osteogenic, chondrogenic, or adipogenic lineages in vitro, a characteristic of MSCs [78]. Rather, most of the CMSCLCs exhibited cardiomyocyte characteristics following ex vivo culture [82]. The therapeutic efficacy of CMSCLCs was not assessed.

Cardiomyocytes and cardiac repair/ regeneration

Bergmann et al. demonstrated that the number of left ventricular cardiomyocyte nuclei remains constant throughout life, even though the size of the heart increases significantly [83]. What is different is the density of these nuclei, which is ~15-fold greater in neonates than later in life. The increased size of the heart and the decrease in cardiomyocyte nuclear density are due to the proliferation of noncardiomyocytes: endothelial and mesenchymal (fibroblasts, pericytes, and smooth muscle) cells [83] and hypertrophy of cardiomyocytes (see Ref. [84], for example). Cardiomyocyte renewal slows down with age and only ~<50% of cardiomyocytes are renewed during a person's lifetime [83]. A consensus was established stating that cardiomyocytes normally undergo mitosis, albeit at a very slow rate, but this rate may increase following injury [85]. The key to cardiac repair is the replacement of lost cardiomyocytes following injury, regardless from where they originate. As previously discussed, the differentiation of cardiac resident progenitors into cardiomyocytes is an infrequent event. Therefore strategies designed to promote endogenous cardiomyocyte renewal appear to be the best approach to promoting cardiac repair. One such approach is cell-based therapy.

Cell-based therapy

Initial studies hypothesized that following injection of stem/progenitor cells post-MI either intramyocardially, or into the coronary or system circulation, the cells would engraft and become cardiac progenitor–like cells and/or differentiate to replace cells (cardiomyocytes, vascular structures) lost to injury. Large animal studies demonstrate that cells that engrafted did exhibit differentiation into cardiomyocytes, endothelium, and vascular smooth muscle cells; however, improvements in cardiac structure and function were incomplete and the amount of engraftment was very low and insufficient to account for the improvements [86,87]. The limited engraftment of transplanted cells led to the hypothesis that the injected cells, whether or not they engraft, secrete factors (secretome) that activate resident cardiac progenitors and cardiomyocytes [88].

The paracrine hypothesis was first proposed by Gnecchi et al. in 2004. They showed that serum-free conditioned media from Akt-overexpressing MSCs had antiapoptotic effects on rat neonatal cardiomyocytes in vitro and reduced scar size within 72 hours of being injected into the myocardium post-MI in vivo [89,90]. This local release of trophic factors includes a wide variety of bioactive compounds such as growth factors and chemo-attractant molecules, as well as exosomes and extracellular vesicles (EVs). Exosomes and EVs are membrane bound micro- or macrovesicles, respectively, which contain mRNA, noncoding RNA (microRNA, etc.), peptides, and other molecules. These secreted molecules are thought to stimulate resident cardiac progenitor cell (CPC) activation, cardiomyocyte proliferation (see Ref. [91] for review), and angiogenesis [92–96], thereby reducing cardiac injury (see Ref. [97]). Exosomes derived from human fetal cardiomyocyte progenitors or adult hBMMSCs were equally effective at stimulating angiogenesis in vivo [98]. CPCs, cardiac fibroblasts, and perhaps even cardiomyocytes release exosomes and EVs that can have beneficial effects on the heart (see Ref. [91] for review).

Recent animal studies have suggested that intact cells or their isolated exosomes produce similar beneficial cardiovascular effects [99] but only when exosomes were injected directly into the myocardium. No improvement

was seen when exosomes were administered via the coronary arteries [100]. Therefore although exosomes represent a cell-free treatment that appears to be therapeutic for MI, additional studies are needed to establish their extent, duration, and the most effective route of administration.

Interaction between cardiac resident and injected cells can also occur through gap junctions [101,102] and "tunneling nanotubes" (TNTs), a process known as heterocellular coupling. Gap junctions are gated channels that allow for the transfer of small molecules (<1000 Da) between cells. This communication is important for coordinating activities between neighboring cells during development and in adult tissues [103,104]. Cells of the myocardium normally communicate with each other through gap junctions as do MSCs that engraft within the myocardium [86]. TNTs are 50–200 nm tubes through which adjacent cells can exchange cell components, including mitochondria and other organelles. The transfer of mitochondria appears to be important for rescuing/repairing injured cells by restoring cellular bioenergetics and oxidative phosphorylation [105,106].

Cardiac progenitor/stem cell therapy

Oskouei et al. directly compared the therapeutic efficacy of human embryonic c-kit$^+$ CSCs and hBMMSCs for cardiac repair in an AMI model in immunodeficient mice. The hCSCs produced greater improvement in hemodynamic parameters and were able to reduce scar size similarly to 30-fold more adult hBMMSCs [107]. Genetically, modifying c-kit$^+$ CSCs to overexpress Pim1 kinase increased their retention within the myocardium and improved their therapeutic efficacy in both a mouse [108] and swine [109] model of acute MI. CSC-derived exosomes recapitulate the major therapeutic effects of CSC administration in both acute and chronic mouse MI models [110]. In a pig chronic MI model, intracoronary delivery of c-kit$^+$ CSCs promoted mitosis in cardiomyocytes. Some of the injected (GFP$^+$) CSCs engrafted and differentiated into cardiomyocytes, vascular smooth muscle, and ECs [111].

While administration of CSCs does promote cardiac repair post-MI, pretreating CSCs with BMMSC-derived exosomes produced greater cardiomyocyte proliferation, migration, and angiogenic potency in vitro [112]. When introduced into the myocardium in a rat model of acute MI, these pretreated CSCs exhibited greater engraftment and promoted greater capillary density, reduced cardiac fibrosis, and improved cardiac outcome than unpretreated cells [112].

ALCADIA (NCT00981006), was an open label, nonrandomized, phase I clinical trial in patients with ischemic cardiomyopathy who underwent coronary artery bypass surgery. This trial addressed the safety and feasibility of intramyocardial injection of autologous c-kit + CSCs in combination with bFGF. Preliminary results showed improvement in left ventricular ejection fraction (LVEF), scar size, and maximal aerobic exercise capacity [113]; however, final results have not been reported.

Combination stem cell therapy

To improve therapeutic efficacy, a novel approach is to combine cells. Small and large animal studies have combined cardiac and extracardiac cells. Quijada et al. created cardiac-chimeras (CCs), a fusion between murine BMMSCs and c-kit$^+$ CPCs, and tested the efficacy of CCs, compared to the combination of BMMSC/CPC or each cell type alone, in a mouse model of AMI. CC-treated animals showed enhanced wall thickness 4 weeks postinjection. At 6 weeks, cardiac function was improved in the CC group, whereas it required 18 weeks for the BMMSC/CPC group. Infarct size reduction and greater persistent engraftment were noted in the CC group compared to BMMSC/CPC [114].

Intramyocardial injection of a combination of BMMSCs and CSCs [cell combination therapy (CCT)] improved cardiac structure and function in swine models of MI. Administration of hBMMSCs and hCPCs (200:1) to immunosuppressed swine 14-days post-MI produced a twofold reduction in scar size, sevenfold enhanced engraftment, and improved LV compliance and contractility 4 weeks later compared to each individual cell type, even though the individual cell types produced significant improvements compared to placebo-treated animals [115]. In a swine chronic MI model, autologous CCT administered 3 months post-MI improved LVEF, stroke volume, cardiac output, and diastolic strain compared to BMMSCs alone. Both cell-treated groups significantly improved scar size, wall motion, and viable tissue compared to placebo [116]. CCT treatment also increased myocardial proliferation compared to placebo (Fig. 31.6). A similar study using allogeneic BMMSCs and/or CSCs again showed that CCT produced greater improvements in cardiac structure and function [117] at least in part by increasing endogenous myocardial cell proliferation [116,117]. Building upon these preclinical large animal studies the ongoing CONCERT-HF (Combination of Mesenchymal and C-kit$^+$ Cardiac Stem Cells as Regenerative Therapy for Heart Failure, NCT02501811) trial, a phase II, placebo-controlled study, is designed to assess if transendocardial administration of autologous CCT with BMMSCs and c-kit$^+$ CPCs provides greater therapeutic efficacy than either cell type alone.

Pluripotent stem cells

Several mouse studies have demonstrated that ESCs can differentiate into functional cardiomyocytes within the host myocardium, improve cardiac function, and prevent

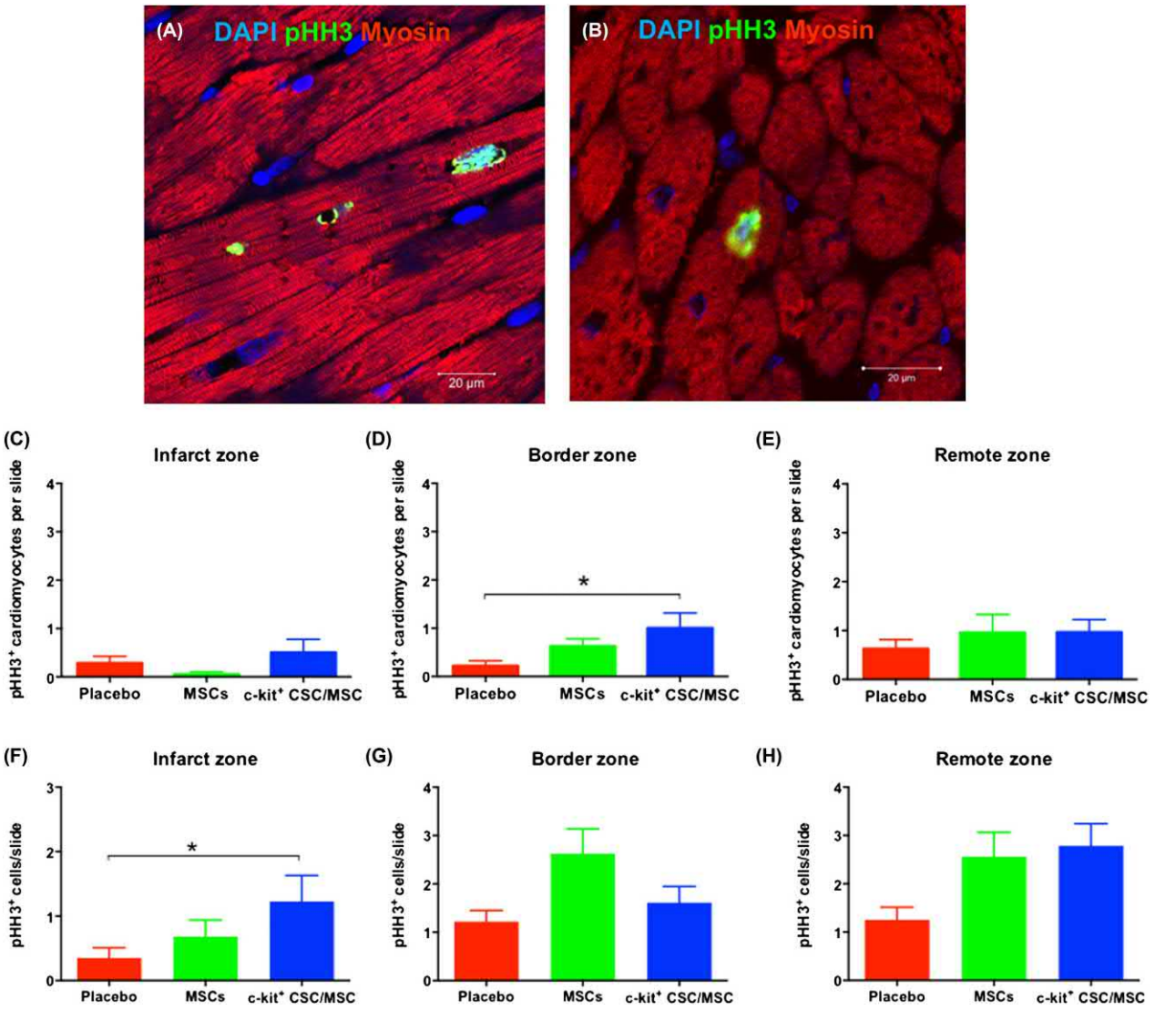

FIGURE 31.6 **Cell treatment–enhanced myocardial mitotic activity.** Pigs received either placebo, autolous MSCs, or autologous CCT. Confocal microscopy depicts increased mitotic activity of endogenous cardiomyocytes [phospho-histone H3–positive (pHH3) nuclei] in (A) border and (B) remote zones in cell-treated hearts 3 months poststem cell administration. Based on the average number of pHH3 mitotic cardiomyocytes per slide per group in the (C) infarct, (D) border, and (E) remote zones, combination cell therapy significantly increased mitotic activity in the border zone compared with placebo ($*P < 0.05$). As depicted, the average number of pHH3 mitotic cells within the myocardium per slide per group in the (F) infarct, (G) border, and (H) remote zones, was increased by CCT which produced significant increases in mitotic cells in the infarct zone compared with placebo. $*P < 0.05$. Red, placebo; green, MSCs; blue, CCT. *CCT*, Cell combination therapy; *DAPI*, 4′,6-diamidino-2-phenylindole; *MSC*, mesenchymal stem cell. *From Karantalis V, et al. Synergistic effects of combined cell therapy for chronic ischemic cardiomyopathy. J Am Coll Cardiol 2015;66 (18):1990–9.*

negative ventricular remodeling after transplantation into the infarcted myocardium [118–121]. However, the pluripotent nature of ESCs risks their developing into teratomas. To circumvent this possibility, more recent studies have focused on pluripotent-derived cells that are more committed toward cardiac lineages. ESC-derived cardiomyocytes (ESC-CMs) express specific cardiomyocyte transcription factors, including Nkx2-5, GATA4, and others [122] and exhibit spontaneous beating activity [123], but these cells remain immature. Similarly, iPSC-derived CMs (iPSC-CMs) are immature [124]. As discussed previously, PSC-derived CMs (PSC-CMs) differentiate into ventricular-, atrial-, and nodal-like cardiomyocytes, and each subtype can be characterized by unique electrophysiological properties [125]. These different types of cardiomyocytes can potentially be used as a therapeutic agent for specific types of cardiomyopathies. Another goal is to direct this differentiation into a more adult cardiac myocyte phenotype with respect to contractility, electrophysiologic performance, and appropriate response to pharmacologic stimulation.

ESC-CMs have demonstrated therapeutic efficacy. Administration of ESC-CMs into post-MI rats improved cardiac function [126] and attenuated the progression of heart failure [127], without the development of teratomas. Furthermore, a comparison of the ability of ESC-CMs, ESC-derived cardiovascular progenitors (ESC-CVPs), and bone marrow–derived mononuclear cells (BMMNCs) to improve cardiac function demonstrated many similarities but also indications that the ESC-derived cell types were similarly efficacious and were generally more therapeutic than the BMMNCs [128].

In nonhuman primate models of MI, hESC-CMs improved cardiac structure and function but produced arrhythmias [129,130]. Another nonhuman primate study demonstrated that ESC-CVP administration improves cardiac function despite an absence of long-term engraftment; a result that supports a paracrine mechanism [131]. Similarly, iPSC-CMs in a nonhuman primate study produced some positive- but also some adverse-effects, including arrhythmogenesis [132]. A direct comparison of hESC-CMs and iPSC-CMs in a nude rat MI model demonstrated that both cell types produced comparable cardioprotection. Furthermore, exosomes derived from these cells contained identical populations of microRNAs and long noncoding RNAs [133]. These studies illustrate the potential of PSC-CMs as a therapeutic agent for cardiomyopathies.

There are few human studies using PSCs. A case study demonstrates that ESC-derived CPCs embedded within a fibrin scaffold and implanted into a patient with severe heart failure improves cardiac function without arrhythmias, tumor formation, or immunosuppression-related adverse events [134]. The ESCORT (transplantation of hESC-derived progenitors in severe heart failure) in which ESC-derived cardiac progenitors were injected into HF patients ($n = 6$) showed safety and efficacy [135].

To date, hiPSC cells have not been tested clinically for heart-related diseases, although based on preclinical studies such studies have been proposed [136]. Transplantation of iPSC-CMs have demonstrated functional benefits in mouse, rat, and pig models of heart disease [137–139]. Furthermore, intramyocardial administration of allogeneic iPSC-CMs post-MI into immune-suppressed, nonhuman primates, resulted in engraftment of the iPSC-CMs and improved cardiac function [132]. However, there was evidence of posttransplant arrhythmias [132]. These results, while promising, require further study to replicate findings and develop countermeasures against malignant ventricular arrhythmias.

Future directions

There is considerable disagreement regarding the role of cardiac progenitor/stem cells in the adult heart. Do they differentiate into cardiomyocytes or support cardiomyocyte proliferation in the healthy and/or injured heart? Can they be manipulated to improve recovery from cardiac injury? PSC models of cardiac development have clarified the role of growth factors in progenitor cell differentiation during cardiac development as have fate-mapping studies in vivo. These approaches, along with newer techniques, including gene editing using CRISPR/Cas9 and in vivo reprogramming of resident cardiac progenitors, cardiomyocytes, and fibroblasts will not only provide answers to these questions but also have the potential for generating an effective therapeutic to reduce the effects of cardiac injury.

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Chapter 32

Cardiac tissue engineering

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Introduction

In the developed world, cardiovascular disease is responsible for the loss of more human lives than all cancer combined. Due to the minimal intrinsic ability of adult heart to regenerate itself following injury [1], the myocardial infarction (MI) results in rapid death of hundreds of millions of cardiomyocytes (CMs), and vigorous inflammatory response. Over subsequent weeks to months, fibroblasts (FBs) and endothelial cells (ECs) form granulation tissue and a dense collagenous scar that reduces the contractile function of the heart and eventually leads to a pathological remodeling and, in many cases, heart failure. Adult CMs are terminally differentiated, thus it is not possible to expand them in vitro to sufficient numbers starting from small cardiac biopsies. Current clinical trials focus on cell replacement through application of bone marrow mesenchymal stem cells (MSCs), peripheral blood mononuclear cells, or resident cardiac cells. Most of these cell types have no intrinsic ability to give rise to a large number of CMs; instead they improve function through paracrine effects. In addition, cells can be applied alone or in combination with different types and forms of biomaterials (e.g., hydrogels and scaffolds). An appropriate combination of a biomaterial, cell type, delivery method, and requirements for tissue culture prior to implantation will depend on the specific type of heart disease and patient population. Here we provide design criteria for the generation of functional cardiac patches and discuss different biomaterials and cell types used during the tissue engineering process.

Clinical problem

Cardiovascular disease is the leading cause of death worldwide. In the United States, cardiovascular diseases account for approximately 30% of all deaths, with cardiovascular disease population constantly increasing [2,3]. In 2008 alone 86.2 million Americans were living with some form of cardiac disease, and this number is projected to grow to 40.5% of the American population by the year 2030 [4]. *Concomitantly*, the financial burden of disease is also projected to rise, from present estimates of $300 billion to $800 billion [4]. On a global scale, cardiovascular disease is responsible for 60% of deaths and will become increasingly important as global obesity and malnutrition continue to rise. Importantly, cardiovascular disease is responsible for 80% of noncommunicable diseases in low- and middle-income countries [5,6], with grave social and economic consequences.

One of the key limitations for the treatment of cardiovascular disease is the lack of regeneration after myocardial injury. The majority of existing therapies aim to mitigate the progression of heart failure, intervening on the cyclic progression of the neurohormonal cascade, but the options for improvement or regeneration of diseased tissue are limited. The most common pathogenesis is the ischemic heart disease, which occurs when a portion of the heart does not receive blood supply of oxygen. Coronary artery disease, or a narrowing of the lumen in the coronary arteries, most often by atherosclerotic change, limits the perfusion of certain sections of the heart. Limiting oxygen delivery past a certain threshold leads to angina, characterized by reversible discomfort or heaviness to the chest. Complete occlusion of an artery, or MI, is associated with a typical pathological progression. Depletion of Adenosine triphosphate (ATP) occurs within seconds, leading to irreversible cell damage by 20−40 minutes [6]. Coagulative necrosis begins ∼30 minutes after coronary occlusion, followed by robust inflammatory response that begins with the release of reactive oxygen species and neutrophil invasion ∼24 hours postinfarction and continues for the next 2−3 days, in parallel to the continued necrosis.

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Macrophages ultimately dominate the infarcted zone by 5−7 days postinfarction and are responsible for removing dead cells and creating granulation tissue. Weeks to months after infarction, collagen deposition dominates, and a fibrous scar is formed [6].

Heart failure, which results in the inability of the heart to adequately pump blood, ensues most commonly after ischemic injury but may also have other etiologies, including valvular disease, hypertension, or genetic cardiomyopathies [7]. An increase in cardiac work requirements leads to one of two patterns of dysfunction in the heart, one where pressure overload dominates, leading to concentric hypertrophy of CMs and a thickening of the myocardial wall, or a second one where volume overload dominates, leading to eccentric hypertrophy of CMs and a thinning of the myocardial wall [6]. In either case, increased fibrosis, abnormal gene expression, and insufficient vascular function lead to pump dysfunction and activation of the neurohormonal system, where the sympathetic nervous tone, renin secretion, and arginine vasopressin secretion all increase. Together, these cues lead to myocardial remodeling at the cellular level that contributes to the progression of heart failure, by mechanisms that are not well understood.

Presently, heart transplant and ventricular assist devices (VADs) can improve cardiac function, but the numbers of available donor hearts are limited, and the VAD is only a temporary solution. Therefore biological treatment strategies that can enhance cardiac function are especially attractive for countering the pathophysiological progression of heart failure. With the advent of induced pluripotent stem cells (iPSCs), there is the newfound promise for cardiac regeneration using patient-specific cells, since CMs were previously unattainable by any other means. Present approaches involve direct cell injection or the creation of a cardiac patch. Cell injections are attractive due to relative simplicity, though poor cell retention is a recurring obstacle [8,9]. A cardiac patch approach would be aimed at replacing or repairing the specific lesion created by MI, using a lab-grown piece of contractile cardiac tissue. Continuing challenges are vascularization and electromechanical integration of such a construct, and these are among the key areas of active research.

Engineering cardiac tissue: design principles and key components

The heart functions as a highly organized physiological pump. The CMs, comprising 80%−90% of the heart volume, are not only elongated and hypertrophied but also aligned and electrically coupled to surrounding CMs. CMs are constantly active, stimulated to beat, and therefore have a high metabolic demand for oxygen. Supporting cell types—ECs and smooth muscle cells—organize themselves into a dense vascular network supplying nutrients to the CMs. FBs support the CMs and generate a collagen-dense matrix. On an organ level, pacemaker cells spontaneously generate action potentials that propagate the volume of the heart, generating a synchronous contraction. The flow of blood through the heart necessitates mechanical stress on the heart, as a preload that stretches the myocardium and afterload to push against.

Fabrication of a functional cardiac patch depends on a multitude of parameters that collectively recapitulate some aspects of the complexity and function of the heart. Since CMs are terminally differentiated, current studies are focusing on deriving a renewable source of CMs from human embryonic and iPSCs. Other studies have aimed at recapitulating one or more physiologic aspects of cardiac tissue (e.g., the incorporation of multiple cell types, aligning CMs, or electrical stimulation of cardiac tissue). Table 32.1 contains a collection of studies that aim to mimic aspects of the native myocardium. The three classical tenets of the tissue engineering paradigm have been used for cardiac constructs: cell source, scaffold materials, and biophysical stimulation (Fig. 32.1).

Cell source

The limited ability of human CMs to divide and expand has restricted the scope and therapeutic potential of cardiac tissue engineering. The first evidence that the application of cells may be a viable therapeutic approach for MI came from animal studies with the injection of fetal or neonatal CMs, wherein CM injection improved left ventricular function and thickness, thus attenuating pathological remodeling upon MI [10−13]. Injected CMs integrated through gap junctions and intercalated disks with the host CMs [14]. However, these findings have limited clinical relevance, and human fetal and neonatal CMs cannot be readily obtained for transplantation due to obvious ethical issues.

The search for a clinically relevant cell source has led to the transplantation of skeletal myoblasts [15], human PSC (hPSC)-CMs [16−18], bone marrow−derived MSCs [19,20], and hematopoietic stem cells [21−23] into animal models of MI (reviewed in Refs. [24,25]). Among these cell sources, skeletal myoblasts and MSCs were pursued into the clinical trials. A metaanalysis of recent clinical trials with injection of bone marrow and peripheral blood mononuclear cells demonstrated a significant, albeit low (3%), increase in left ventricular ejection fraction (LVEF) as well as a significant reduction in infarct size (−5.6%) and end-systolic volume (−7.4 mL) in patients treated by intracoronary cell injection after acute MI [26].

TABLE 32.1 Design parameters for cardiac tissue engineering.

Native cardiac attribute	Engineering method	Results	References
Cell source	Sequence of activin and BMP4 cytokines induces cardiac cell differentiation in human embryonic and iPSCs	Flow cytometry at multiple timepoints revealed high sensitivity of multiple Embryonic Stem Cell (ESC) and iPSC lines to concentrations of induction factors. However, optimization of the times and concentrations lead to improved differentiation yields	Kattman et al. [77]
	Lentiviral iPSC technology using epigenetically specific cell sources, ventricular cells versus fibroblasts	Ventricular cells as material for iPSCs improved cardiac differentiation potential when compared to using fibroblast cells, suggesting epigenetic memory for iPSC lines	Xu et al. [78]
Patient cell source	iPSCs were derived from fibroblasts of patients with long QT syndrome type I using infection with retrovirus encoded with Oct3/4, Sox2, KLF4, and c-MYC. They were subsequently differentiated into CMs	Generation of iPSCs was confirmed with Nanog staining, and generated CMs showed striations when stained with cardiac troponin T. LQT1 disease phenotype of lengthened ADP 50 and ADP 90 was confirmed by single-cell patch clamp. Myocytes generated from LQT1 patients showed KCNQ1 channel localization to the endoplasmic reticulum, suggesting a possible mechanism for the disease	Moretti et al. [79]
	IPS cells derived from patients with familial DCM were generated using Oct4, Sox2, Klf4, and c-MYC. They were subsequently differentiated into CMs using the protocol developed by Yang et al. [32]	Immunofluorescence images of Oct4, Nanog, TRA-1−81, and SSEA-4, as well as hypomethylation as seen by quantitative bisulfite sequencing confirmed the generation of iPSCs. Multielectrode array analysis of DCM-derived CMs displayed similar beat frequency, interspike intervals, and field potential durations as the control group. Immunohistochemistry and TEM displayed similar cell size, but DCM-derived cells had a higher relative percent of disorganized cells based on α-actinin staining. Overexpression of Serca2a resulted in restoration of contraction force as measured by Atomic Force Microscope (AFM). Treatment of cells with β-blockers decreased the percentage of disorganized cells	Sun et al. [80]
Multiple cell types	Porous PGS scaffolds were preseeded with cardiac fibroblasts encapsulated in Matrigel for 5 days and were subsequently seeded with CMs for another 5 days. This was compared to constructs of cardiac fibroblasts coseeded with CMs	Immunofluorescent analysis of constructs preseeded with fibroblasts showed a greater percentage of actin-positive cells compared with vimentin-positive cells, and compared with actin-positive cells in the coseeded group. The preseeded group further showed lower excitation threshold, higher fractional area change, and increased fluorometrically measured DNA and protein content	Radisic et al. [46]
	Endothelial cells were cocultured in cell sheets with CMs at different ratios	Higher seeding ratios of endothelial cells resulted in higher density of vascular networks as appreciated by fluorescent images. ELISA for secreted factors showed higher levels of VEGF, basic fibroblast growth factor (bFGF), and Hepatocyte growth factor (HGF) in endothelial coculture groups compared to CMs alone. Fluorescent views of implanted cocultured cell sheets showed neovascularization into the myocardium	Sekine et al. [47]
	External endothelial cell addition to cardiac tissue constructs	Increased CM DNA content demonstrated a 35% increase in CM proliferation due to addition of endothelial cells, validating coculture effects of endothelial cells. In addition, vascular engraftment was shown when implanted with blood vessels of patient	Tulloch et al. [43]

(*Continued*)

TABLE 32.1 (Continued)

Native cardiac attribute	Engineering method	Results	References
High cell density	Cell sheet method, where confluent layers of cells are detached from a temperature-dependent poly(*N*-isoproplyacrylamide) substrate and stacked on top of other cell sheets using a cell sheet manipulator. Cell sheets were compared to cell injection in an infarct model	There was a greater in vivo bioluminescence of implanted green fluorescent protein (GFP)-expressing cell sheets compared to injected GFP cells. Macroscopic fluorescent views of cell sheets versus injected cells showed dense localization 4 weeks posttransplantation. Immunohistochemistry showed a greater density of cells. TUNEL staining showed a significantly lower level of TUNEL-positive nuclei compared to cell injection	Sekine et al. [9] Haraguchi et al. [68]
Ultrastructure	Microcontact printing of fibronectin into rectangles of various aspect ratios and seeded with CM pairs	Connexin-43 immunosignal and conductance as measured with dual-voltage clamp was greater in the 5.2 length:width ratio as compared to 3.5	McCain et al. [81]
Alignment/ anisotropy	Soft lithography of PGS into accordion-like honeycombs of overlapping diamonds	Differential long and short-axis elastic moduli and excitation threshold that mimic the anisotropy of the heart. Immunofluorescent actin images demonstrating elongation in the preferred direction	Engelmayr et al. [56]
	Polydimethylsiloxane (PDMS) microcontact printing of fibronectin into shapes of various aspect ratios	Image analysis of immunofluorescence actin images demonstrated greater anisotropy in shapes with >2:1 aspect ratio as compared to the 1:1 group	Bray et al. [61]
Extracellular Matrix (ECM)	Neonatal hearts were decellularized with antegrade coronary perfusion of SDS and were recellularized with neonatal CMs, fibrocytes, endothelial cells, and smooth muscle cells	Decellularization and recellularization were confirmed by histological analysis and staining. The perfused recellularized heart showed synchronous contraction as measured by ECG and left ventricle pressure (LVP) after electrically stimulated depolarization. Ejection fraction totaled 25% of an equivalently aged healthy fetal heart	Ott et al. [49]
	Decellularized sheets of human myocardium were used as a scaffold for human mesenchymal progenitor cells suspended in fibrin and implanted into infarcted rat hearts	Histological staining of ECM proteins and tensile testing showed that decellularized tissues were similar to native tissue. Echocardiographs performed on constructs transplanted on rat hearts preserved left ventricular systolic area and fractional area change	Godier-Furnemont et al. [50]
	PGS scaffolds were prepared at different stiffnesses by altering the curing time and were subsequently seeded with neonatal rat CMs	Low stiffness groups were found to have the greatest functional change (contraction amplitude) and also the greatest compressive stiffness	Marsano et al. [82]
	Comparison between collagen/Matrigel mixture with and without fibrin	Fibrin enhances the CMs alignment	Zhao et al. [83]
Cardiomyocyte hypertrophy	Molded rings of neonatal rat CMs, Matrigel, and collagen I and cultured in different hypertrophic stimuli (angiotensin II and phenylephrine versus hypertrophic inducing serum) on a cyclic stretch device for 12 days	Immunofluorescent analysis of single cells in the angiotensin II and phenylephrine group showed no change in length but displayed increased width and total volume suggesting concentric hypertrophy. Similar analysis on the "hypertrophic inducing serum" group displayed significant elongation without a widened morphology suggesting eccentric hypertrophy. Further evidence for hypertrophy in both groups is supported by high levels of gene expression of atrial natriuretic peptide (ANP) and low levels of gene expression of α/β-MHC	Tiburcy et al. [54]

Electrical stimulation	Carbon electrodes field stimulation	Ultrastructurally improved contractile apparatus and gap junctions. Histology and immunostaining showed increased linear organization. Contracting force increased and improved electrical maturity	Radisic et al. [74]
	Gold nanowire impregnated alginate scaffolds	Electrical conductance through gold impregnated scaffolds was increased. Connexin-43 expression was doubled compared to nonimpregnated controls	Dvir et al. [84]
	hESC CMs transplanted in ablated large animal heart model	ECG mapping colocalized injected CMs to ectopic ventricular pacing, demonstrating pacing potential of injected hESC pacemakers	Kehat et al. [85]
	Advanced functional maturation through progressive electrical stimulation	Extensive functional maturation with adult-like gene expression profiles, remarkably organized ultrastructure, physiological sarcomere length (2.2 μm) and density of mitochondria (30%), the presence of transverse tubules, oxidative metabolism, a positive force–frequency relationship, and functional calcium handling	Ronaldson-Bouchard et al. [86]
	Chamber-specific cardiac tissue maturation via chronic electrical stimulation	Chamber-specific tissue maturation in terms of electrophysiology, genetic profile, structural and functional protein expression, and chamber-specific drug responses	Zhao et al. [87]
		Specifically designed 8-month electrical conditioning simulates the chronic cardiac workload, and allows manifestation of disease phenotypes (left ventricular hypertrophy) in vitro	
Vascular perfusion	Perfusion bioreactor through channeled scaffolds	Perfused channeled scaffolds showed nearly 50% increase in viable cells compared to nonperfused controls. Finite element modeling provides a rational approach for vascular perfusion design through engineered cardiac tissue	Radisic et al. [63]
	Omental prevascularization of cardiac patch	Prevascularization improved engraftment on the infarcted heart and mitigated decline in cardiac function based on echocardiography	Dvir et al. [84]
	Multilayer prevascularized cardiac patch	Built-in hierarchical vasculature made of biodegradable scaffold allowing extensive perfusion and direct surgical anastomosis	Zhang et al. [67]
Force generation	Engineered heart tissues, ring-shaped CM aggregates in a mixture of Matrigel and collagen, were placed onto load-adjusted coils to apply a passive, auxotonic load on the tissues	Engineered heart tissues on auxotonic load displayed a greater twitch tension by isometric force contraction analysis. Four weeks after implantation onto an infarcted rat heart, echocardiographs, MRI, and catheterization together demonstrated decreased left ventricular volumes, lower left ventricular end-diastolic pressures, and shorter relaxation times when compared to sham-operated rats	Zimmerman et al. [55]
	CMs in porous chitosan–collagen scaffolds were stretched by moving four pins on the scaffold. Nominal strain of approximately 20% were applied at a frequency of 1 Hz for 6 days	Regions of high local stress were determined using a mathematical model. Immunohistochemical analysis showed high levels of connexin-43 staining at the regions of high stress. Histological analysis and scanning electron microscopy (SEM) also demonstrated elongated morphologies in areas of high stress in comparison to areas of lower stress	Zhang et al. [58]
Patch assembly, delivery, and integration	An elastic and microfabricated scaffold based on a biodegradable polymer [poly (octamethylene maleate (anhydride) citrate)] for functional tissue delivery via injection	The patches significantly improved cardiac function following myocardial infarction in a rat, compared with the untreated controls. Successful minimally invasive delivery of human cell–derived patches to the epicardium, aorta and liver in a large animal (porcine) model was achieved	Montgomery et al. [88]
	A bio-scaffold with a microfabricated hook and loop system allowing rapid integration of a large functional cardiac patch	The technology enables on-demand tissue disassembly while preserving the structure, physical integrity, and beating function of individual layers. The technology also allows instant establishment of coculture conditions by spatially defined stacking of cardiac cell layers or through endothelial cell coating	Zhang et al. [65]

BMP4, Bone morphogenetic protein 4; *CMs*, cardiomyocytes; *DCM*, dilated cardiomyopathy; *ECM*, extracellular matrix; *iPSCs*, induced pluripotent stem cells; *SDS*, sodium dodecyl sulfate; *TEM*, transmission electron microscopy; *VEGF*, vascular endothelial growth factor.

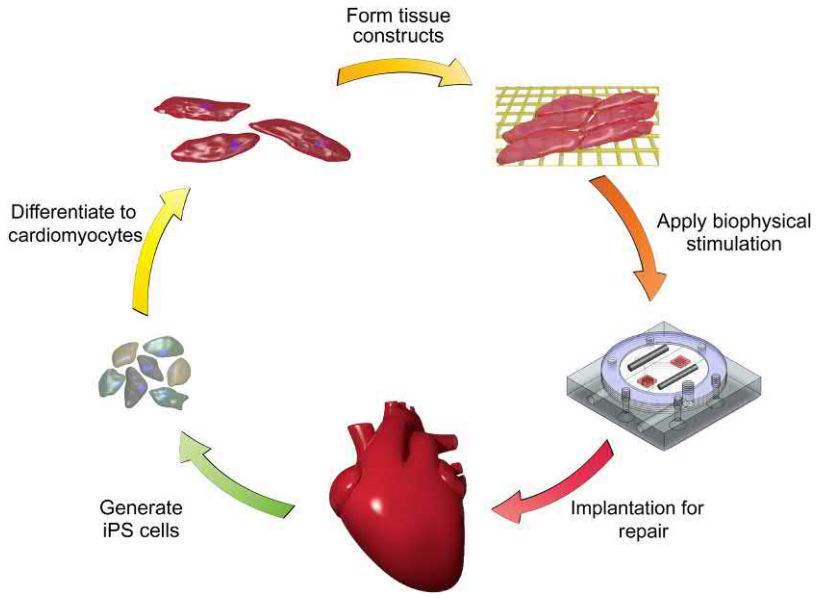

FIGURE 32.1 Cardiac tissue engineering approach.

A dose–response effect of the injected cell volume on LVEF change was also reported [26]. During the last year, resident cardiac stem cells, either c-kit + (SCIPIO) or those derived from cardiospheres (CADUCEUS) demonstrated promising functional improvements in Phase I clinical studies and restoration of viable tissue per MRI imaging, presumably due to the new CMs in addition to vascular cells [27,28].

Although these studies are encouraging, modest long-term improvements in function upon cell injection have motivated the investigation of new cell sources and methods that increase survival and retention of injected cells. Pluripotent stem cells such as *human embryonic stem cells* (hESCs) or *iPSCs* can both give rise to bona fide CMs and be expanded to sufficient numbers (millions/patient) using existing technologies. The discovery of human iPSCs (hiPSC) [29] and the ability to generate CMs from them [30] could provide effectively unlimited numbers of autologous CM for cell therapy without the ethical concerns raised by the use of hESC. Studies from a number of groups have shown that it is possible to generate CMs from mouse [31] and hESCs [32] and iPSCs [30]. With years of optimization the cardiac differentiation protocols can provide billions of CMs [33] in an efficient manner by regulating lineage commitment in the early embryo [32]. The protocols are now being adapted to defined culture conditions, with the use of small molecules such as glycogen synthase kinase 3 inhibitors and chemical inhibitors of wingless-type MMTV integration site family (WNT) signaling [34].

While in many ways iPSC-derived CMs are ideal, the exact approach for their utilization is not entirely clear. Though the differentiation process has become more specific than previous work relying on stochastic cell differentiation, further refinements are possible and necessary. In particular, there are multiple phenotypes of CMs, each with a different set of functions [35]. To be more specific, atrial, ventricular, and pacemaker cells are three major subtypes of CMs. With differentiation protocol refinement, chamber-specific atrial and ventricular CMs [36] as well as pacemaker cells [37] can be obtained in high purity. These cells can then be used for the construction of chamber-specific cardiac tissues. Shiba et al. recently demonstrated conclusively that hESC-CMs can electrically couple and suppress arrhythmias in hearts upon MI induced by cryoinjury [38]. As cell death is a major problem for cell injection studies—with up to 90% injected cells dying or being washed away from the injection site—the authors used a prosurvival cocktail consisting of Matrigel and various growth factors to enhance cell persistence upon injection [39].

Another issue is the cell maturity [40–42]. Being recently differentiated, iPSC-derived CMs are relatively young, and further phenotypic maturation may be needed to grow them into adult-like myocytes with their inherent contractile properties. Furthermore, even after differentiation and maturation, a pure population of CMs would not be most apt for building cardiac tissue. Coculturing with nonmyocyte populations, such as cardiac FBs, ECs, and smooth muscle cells, has been shown to effectively enhance tissue function, mimicking the heterogeneity of cells found natively [43–48]. The cell-related parameters of interest for building a cardiac patch include cell identity that accounts for specific CM type, relative ratios of different cells, cell density, and cell maturity.

Scaffold

Scaffolds provide a three-dimensional (3D) environment in which the cells are cultured. The constituents and

organization of the scaffold dictate the organization, maturation, and function of the forming tissue constructs. The scaffold material itself ranges from the native heart matrix itself [49–51] to natural hydrogels such as collagen, fibrin, and Matrigel [8,52–55] to synthetic polymers, such as poly(glycerol sebacate) (PGS) or polyacrylamide [56–58]. The scaffolds differ in the way they are processed (e.g., decellularization for native heart matrix versus temperature-related gelation for Matrigel), their mechanical properties, their ultrastructure, and their biodegradability. With the wide library of materials to choose from, scaffolds can be made into virtually any size or shape, depending on the application. Micropatterning has been employed as a way to control CM alignment and cell–cell interactions on a single-cell level [48,59–61], while macrosized constructs offer amenability to force generation and animal implantation studies [39,48,62].

Due to the large metabolic demand of CMs, scaffold design must take oxygen and nutrient delivery into account. The cardiac tissue engineering scaffolds are in general porous and perfusable to enable oxygen supply in vitro [41,63–65] and designed to promote angiogenesis to enable oxygen supply in vivo [62,64,66,67]. Some methods of cardiac tissue engineering do not utilize scaffolding material. For example, the cell sheet method [9,68–70] relies on stacking confluent sheets of CMs and the extracellular matrix (ECM) these cells alone produce.

Biophysical stimulation

Further phenotypic maturation of cardiac constructs can be achieved using biophysical stimulation. Perfusion of engineered constructs helps alleviate the diffusional limitations [62–64,67], which is of particular importance for the highly metabolically active cardiac tissue. The flow of medium across or through the engineered construct mimicking the vasculature found in native tissue to provide fresh media and nutrients while removing toxic metabolic products from the cells. These approaches are critically important for the generation of large and thick, clinically relevant sized cardiac constructs with homogenous distributions of cells.

Other stimulation modalities rely on the excitation–contraction coupling property that is inherent to heart tissue [71]. Stimulation systems can excite cells using either electrical depolarization, mechanical strain, or the combination of both. Mechanical stimulation systems use active or passive tension enhancing cellular organization, morphology, and contractile force generation. Auxotonic systems maintain passive tension on engineered cardiac constructs, providing a tonic resisting force for the cells to pull against [55,72]. Phasic systems provide active, cyclic strain to improve cardiac function, though some studies have shown improved twitch forces created using the auxotonic method [43,55,58]. Electrical field stimulation uses electrodes to provide a depolarizing stimulus [42,64,73–76]. The electrical field that excites the cells of the construct is created by a voltage difference across the two electrodes. This cyclic depolarization improves electrical synchronization of cardiac constructs, while also improving contractile function and cellular organization. Both of these modalities of biophysical stimulation lead to functional enhancements such as improved CM ultrastructure, improved sarcomeric linearization and organization, and increased functional gap junctions.

Directed cardiac differentiation of human stem cells

Cardiac tissue engineering requires a reliable source of CMs. Human adult CMs are unsuitable as they do not have the ability to proliferate. Recent advances in hESC and iPSC technologies have allowed for the generation of human CMs from healthy progenitors as well as from diseased individuals. We describe here the differentiation of CMs from an ESC-derived progenitor. We further discuss the purification and characterization of CMs, as well as current efforts to generate patient-specific CMs.

Derivation of cardiomyocytes from human pluripotent stem cells

One of the first accounts of generating human CMs from an ESC source comes from Yang et al. [32]. This study focuses on the discovery of a common cardiovascular progenitor capable of generating the different cell types dominant in the adult heart, that is, CMs, ECs, and smooth muscle cells. This method uses a combination of several factors to recapitulate cardiac development (Fig. 32.2A and B), resulting in a large percentage of troponin-positive cells (Fig. 32.2C). The ESC cultures were first induced with activin A and bone morphogenetic protein 4 (BMP4), generating a $T+$ population, or one which overexpressed brachyury, an important transcription factor defining the primitive streak and mesoderm. The cultures were next induced with Dickkopf-related protein 1 (DKK1), a canonical WNT inhibitor, and vascular endothelial growth factor. DKK1 was necessary for generating CMs, as demonstrated by a subsequent increase in ISL1, a marker for the secondary heart field, and NKX2.5, a marker for cardiac differentiation, and increases in cardiac-specific proteins MLC2A and cardiac troponin T.

Interestingly, flow cytometric data of embryoid bodies at various stages of cultivation demonstrated three distinct populations at day 6 of induction (Fig. 32.2D). Stage I corresponds to a stem cell growth factor receptor

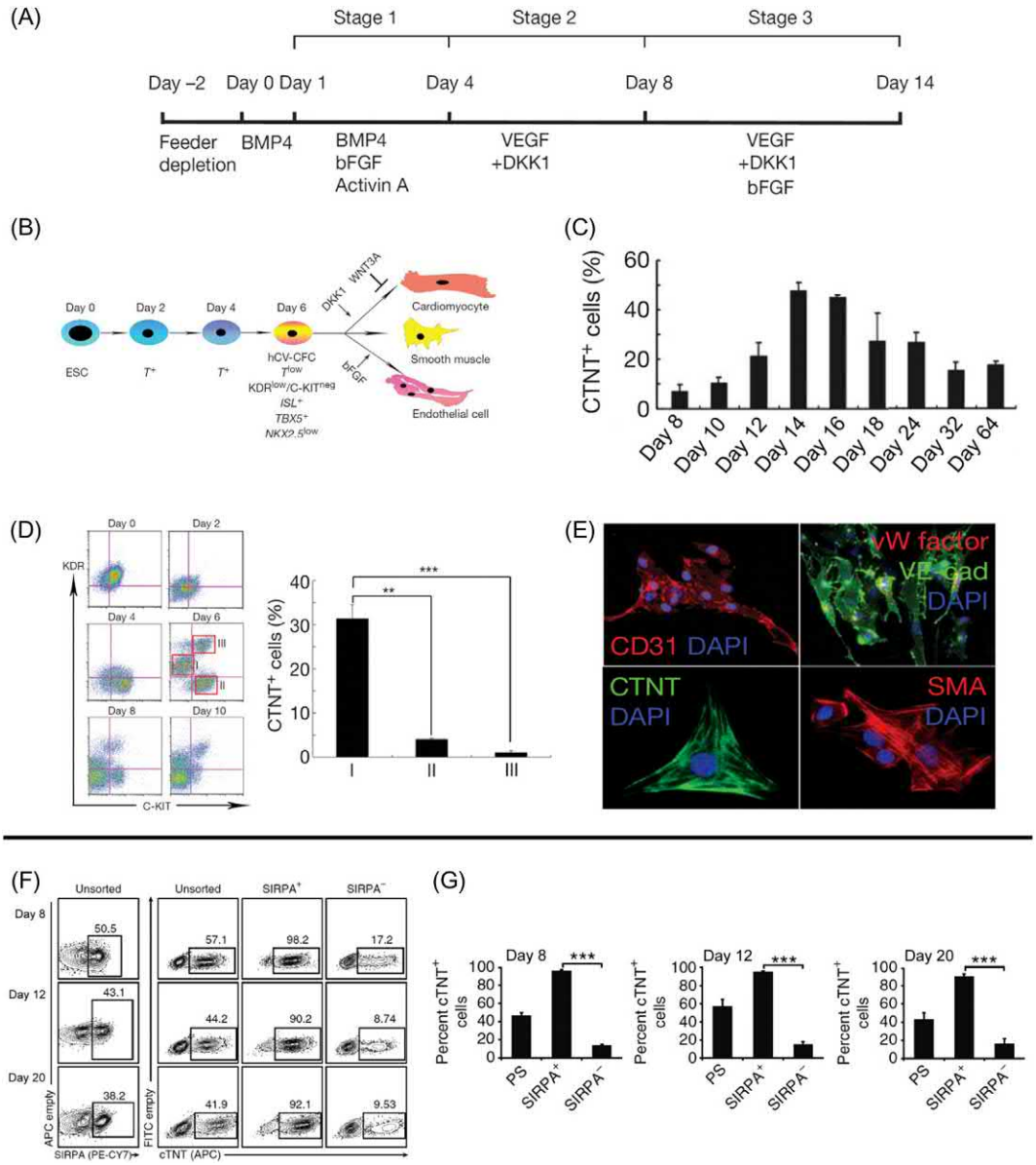

FIGURE 32.2 Generation of cardiomyocytes from embryonic stem cells [32]. (A) Cardiomyocyte derivation protocol and (B) corresponding schematic of development of cardiovascular lineages. (C) Percentage of troponin-positive cells over time using the protocol in (A). (D) Flow cytometry reveals three populations on day 6 of differentiation KDR^{low}/C-KIT^{neg} (I), KDR^{neg}/C-KIT^{pos} (II), and KDR^{high}/C-KIT^{pos} (III), where differentiation of the KDR^{low}/C-KIT^{neg} population yielded the highest percentage of troponin-positive cells. (E) Immunostaining for endothelial cell markers CD31 and vWF, cardiomyocyte marker cTNT, and smooth muscle marker SMA on cells differentiated from the KDR^{low}/C-KIT^{neg} lineage. (F) Flow cytometric analysis of hESC-derived cardiomyocytes sorted for SIRPA on various days (8, 12, and 20) of differentiation. (G) SIRPA positivity selects for a cardiomyocyte progenitor, resulting in up to 98% troponin-positive cells. *hESC*, Human embryonic stem cells; *SIRPA*, signal regulatory protein alpha.

(KDR)^{low}/stem cell growth factor receptor (C-KIT)^{neg} population, stage II corresponds to a KDR^{neg}/C-KIT^{pos} population, and stage III corresponds to a KDR^{high}/C-KIT^{pos} population. KDR and C-KIT were chosen for further investigation based on their significance in mouse stem cell studies. In particular, KDR was shown in mice to give rise to cardiac progenitor, and C-KIT was shown to derive hematopoietic and vascular lineages [31]. Low KDR expression and C-KIT positivity defined a cardiac progenitor, developing into cells with a greater number of cardiac troponin-positive cells (>50%) compared with the other subpopulations (Fig. 32.2D). As lineages of cardiovascular cells are induced, significant populations of CMs, ECs, and smooth muscle cells form in culture (Fig. 32.2E). Transplantation of these progenitors into murine hearts has led to differentiation into the same three cell types—CMs, ECs, and smooth muscle cells—and resulted in an increase in ejection fraction (56% vs 39%).

Whole-cell patch clamp and microelectrode arrays of the CMs demonstrated results consistent with cardiac phenotype. Together, these results suggest the identification of a cardiovascular progenitor that gives rise to CMs, ECs, and smooth muscle cells.

Furthermore, Lian et al. have demonstrated that cardiac differentiation can routinely achieve high (up to 98%) purity and high yield (15 CMs from 1 PSC) through precisely regulating Wnt signaling pathways with small molecules (CHIR99021 and IWP4) or a genetic approach [34,89]. This robust, inexpensive, completely defined, growth factor−free scalable method of producing CMs can be achieved for multiple hPSC cell lines and has been widely adapted by several cardiac-related research fields [83,86,87,89,90].

CMs have many subtypes. CMs from atrial, ventricular chambers, and Sinoatrial (SA) nodal area have distinct action potential profiles, different protein expression, and differential drug responses [87]. Therefore it is important to obtain the CM subtypes at a high purity for better chamber-specific functional tissue constructs. The studies of mouse and chick embryos have revealed that retinoic acid (RA) is a key regulator of cardiovascular fate [91,92]. Addition of RA in a specific time window is a key to differentiate chamber-specific CMs subtypes, such as atrial and ventricular CMs. Lee et al. extensively evaluated the experimental outcomes and precisely defined the atrial-specific differentiation protocol, which includes the addition of 500 nM RA at the beginning of the mesoderm induction (day 3−12 after start of the CM differentiation) [36]. Without further purification, the percentage of atrial CMs, defined as $MLC2v^{neg}/cTNT^{posi}$, was above 80% [36]. Portze et al. developed the first SA nodal−specific differentiation protocol. hPSCs were first induced to mesoderm with a combination of bFGG, low amount of activin A and BMP4. The cells then went through inhibition of bFGF, TGF-β, and Wnt signaling in the presence of BMP4 and RA [37].

Purification and scalable production of stem cell−derived cardiomyocytes

While this original study and subsequent studies have identified reproducible ways of generating cardiovascular lineages with high purity and high yield [93], one major challenge was to completely separate the CMs from the nonmyocyte cell types, necessary to provide more defined cellular compositions for cardiac tissue constructs. In a screen for a marker of hESC-derived CMs, signal regulatory protein alpha (SIRPA) appeared to specifically select for CMs above the other cell types (including ECs and smooth muscle cells) [94]. Indeed, cell sorting with this marker and depletion of ECs and smooth generation of a pure cell source is of particular importance since the implantation of undifferentiated cells may lead to teratoma formation. Another purification method is through the unique metabolic activity of CMs [95]. Burridge et al. replaced glucose in the media with lactate to realize metabolic purification. By eliminating the glucose in the media, only CMs in the culture are able to use the tricarboxylic acid cycle (TCA) cycle to produce ATP and other cell types are starved to death. In this case the CM purity after metabolic selection is >90% based on TNNT2 flow cytometry [95].

Given the precision required to generate specific cell types from stem cells, protocols must be optimized. For example, the procedure originally proposed by Yang et al. has been further modified and applied to multiple cell lines, including iPSCs [77]. Use of micro-bioreactor arrays allows controlling over the 3D cellular microenvironment in a multiplexed fashion, toward experimental optimization of cell derivation procedures on a small scale [96]. In addition, these devices allow controlling more than simply the concentration or type of cytokines.

The small molecule differentiation protocol enables scalable CMs differentiation process. Tohyama et al. recently demonstrated the ability to differentiate hPSC-derived CMs in monolayer culture in 10-layer, 1.2-L culture flasks with active gas ventilation, creating near a therapeutically relevant number of $1.5-2.8 \times 10^9$ cells with >66% purity [97]. Chen et al. seeded undifferentiated hPSC aggregates in reactors to scale up the production of hPSC-derived CMs in 3D suspension culture. The reactor can yield 1.5−2 billion CMs in a 1 L spinner flask with >90% purity [98].

Scaffolds

Cells alone do not compose functional tissues; other critical components include substrate and ECM that surrounds and instructs the cardiac cells. Natively, the ECM provides microenvironmental cues, mechanical support, and architectural guidance, acting as the scaffold upon which cardiac cells grow and function. Previous tissue engineering scaffolds have used polymeric materials, lyophilized collagen sponges, and micropatterned anisotropic materials. These artificial materials have design advantages in that they can be microfabricated, functionalized and are highly reproducible, but other approaches have used decellularization of native matrix as a different starting point, providing scaffold with improved biological activity at the cost of some of the versatility of synthetic approaches.

Decellularization approach

Decellularization of native heart material is a powerful approach to easily recapitulate the in vivo architecture

and extracellular composition of the heart. In 2008, Ott et al. decellularized whole rat hearts using a perfusion system of 1% sodium dodecyl sulfate in deionized water as shown in Fig. 32.3A [49]. The subsequent washes of detergent removed all of the cellular components from the native heart leaving behind a "ghost" heart, translucent in appearance, composed on thin walls of native heart matrix, which retained macro- and microscopic architecture.

When reperfused with a native blood supply, the decellularized hearts clearly showed maintenance of vascular channels, demonstrating the preservation of overall native morphology (Fig. 32.3B). When reseeded with neonatal rat cells, the heart regained a cellularized appearance, and sections cut from the reseeded heart were capable of beating and matched the pacing rate applied through the external electrical field, either at 1 or 2 Hz (Fig. 32.3C). Over the 8 days of culture, there was an increasing trend of contractile function and pressure generation. The whole heart preparations were also exposed to phenylephrine, which increased contractile pressures, suggesting pharmacological responsiveness (Fig. 32.3D). Overall this study provided a basis for whole heart decellularization with subsequent repopulation, providing a cardiac pump function 2% of the adult rat heart output.

Since cardiac MIs are localized as compared to the whole heart, sometimes only a specific section, importantly the left ventricle may need to be the target of therapy [50]. Decellularized scaffold patches can be made from native human heart sections using sequential detergent washes. However, the direction of sectioning is important, as different planes of section result in different scaffold architectures and different pore sizes (Fig. 32.3E).

Alternatively, decellularized cardiac tissues and other tissue types can be digested into gels and used as injectable scaffolds loaded with CMs or other cells [99]. Despite losing intrinsic architecture of decellularized ECM, injectable hydrogels are touted for their minimally invasive delivery, ability to self-assemble in situ, and capacity to encourage host tissue regeneration. The material has since shown efficacy to increase cardiac muscle, reduce fibrosis, and improve cardiac function post-MI in small and large preclinical animal models [100,101].

When vascular progenitor human MSCs are seeded onto these native scaffolds, they act as depots of vasculogenic factors improving recovery of left ventricular function damaged by infarction. As shown in Fig. 32.3F, the presence of infarct improved migration of the vasculogenic MSCs into the damaged tissue, in both acute and chronic models, and suggested that the stimulus of injury improved the responsiveness of cardiac tissue to the therapeutic cells. Notably, cells did not stay at the surface epicardium of the heart, and instead, they penetrated into the myocardium even when only microinfarcts were created using sutures alone to damage the myocardium. These native cardiac patches acted as a vehicle for a vasculogenic cell–based therapy and showed improved heart function, using echocardiographic metrics such as fractional shortening and fractional area change of the regions infarcted (Fig. 32.3G). The use of the native matrix scaffold improved recovery above cell injection alone proving additional benefit as a delivery vehicle for the vasculogenic cells.

Artificial scaffolds

Despite advances in using native matrices as scaffolding agents for the delivery of cells to injured heart, rational design of artificial scaffolds is still an active area of research. One primary limitation of fabricated scaffolds was the lack of electrical conductivity, a property that might enhance cell–cell communication and synchronization of the heart. Dvir et al. used gold nanowires impregnated in a conventional scaffold material, alginate, to bestow conductive properties to the material [84]. Fig. 32.3G outlines how conductive scaffold allows electrical signal propagation. Since alginate was chosen as the base scaffolding material, it maintained many of its familiar features. Even with the addition of gold nanowires (Fig. 32.3H), the viscosity and material properties of alginate were not altered enough to change the porosity of the final scaffolds. The presence of the nanowires improved expression and organization of connexin-43, the primary gap protein associated with electrical–mechanical coupling and communication between cells, assessed using immunofluorescent staining and western blot quantification (Fig. 32.3I). When calcium transients were investigated, the nanowire impregnated scaffolds showed fluorescent signal consistent with electrical propagation through the scaffold material, providing electrical stimulation to the cells. Similarly, carbon nanotubes can also be incorporated into the scaffold material (1,2,4 polymer elastomer) at 0.5 wt.% to improve the electrical conductivity, which eventually enhanced the functional maturity of the cardiac tissue [102]. Thus engineered materials can incorporate unique properties to cardiac scaffolds, such as mechanical properties [65,67], conductivity [102], topographical niche [65,67,88,103], and tailor their functionality to that found in the native heart muscle.

Engelmayr et al. created an accordion-like scaffold using laser boring of 250 μm thick PGS layer [56]. The accordion-like honeycomb was made by overlapping two 200 by 200 μm^2 at the angle of 45 degrees. The pore walls and struts were ~50 μm thick. The scaffolds were pretreated with cardiac FBs followed by seeding of enriched CMs. At the end of cultivation the authors obtained contractile cardiac grafts with mechanical properties closely resembling those of the native rat right

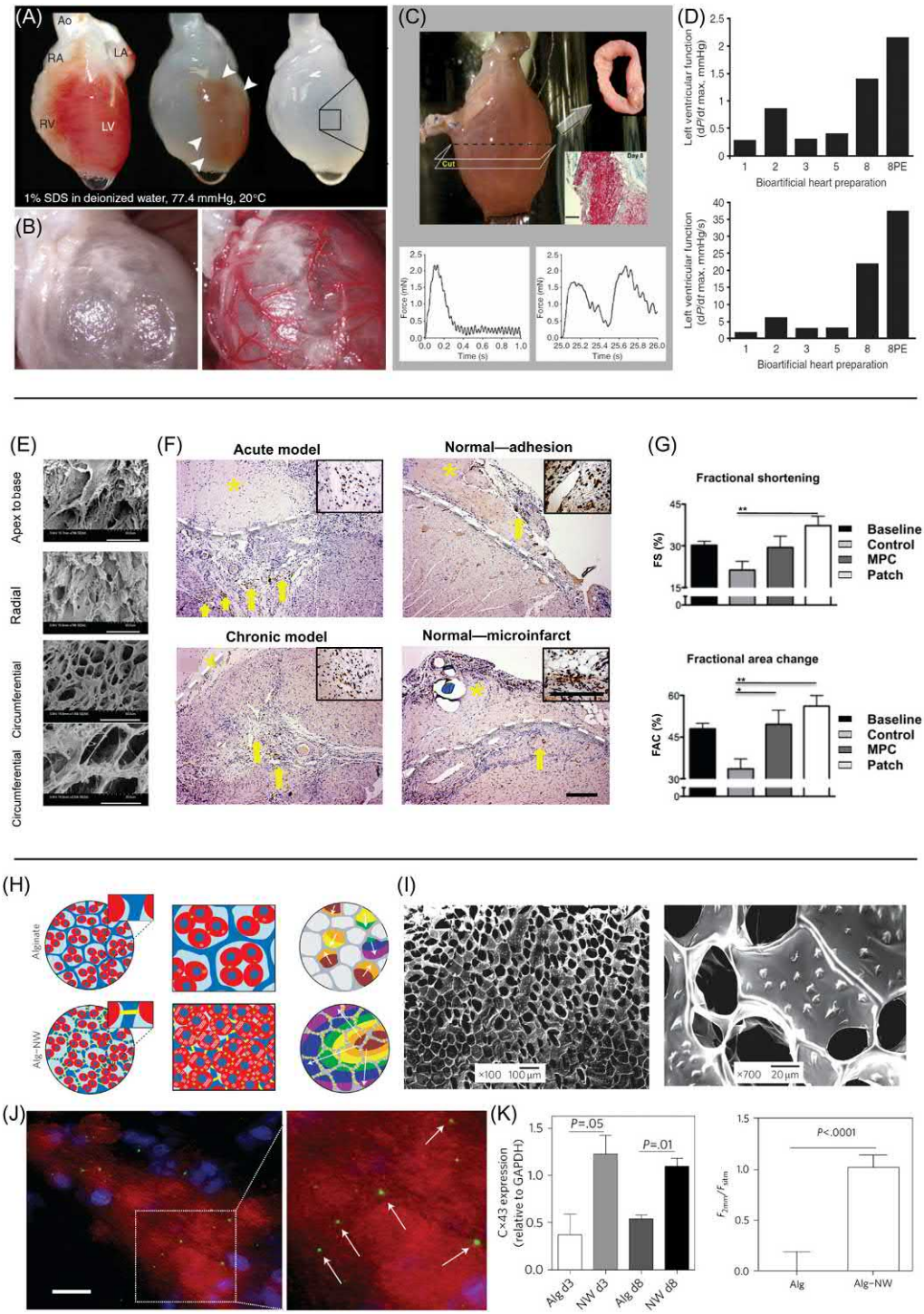

FIGURE 32.3 **Scaffolds for cardiac tissue engineering.** (A) Decellularization of whole neonatal heart with 1% SDS over the course of 12 hours [49]. (B) Perfusion of the decellularized heart with the host vasculature through the aorta demonstrates maintenance of the decellularized blood vessels [49]. (C) Hearts recellularized with cardiomyocytes beat spontaneously after 4 days in culture and can generate force when paced at 1 or 2 Hz [49]. (D) Maximum pressure and d*P*/d*t* after 8 days in culture and after stimulation with PE [49]. (E) Scanning electron microscope images of various slices of heart yield different pore sizes [50]. (F) Migration of human mesenchymal progenitor cells (MPCs) from MPC/scaffold/TGFβ constructs to acute infarcts, chronic infarcts, or normal myocardium 4 weeks after implantation [50]. (G) Fractional shortening and fractional area change for 3 days postinfarction rats (baseline), which were subsequently stratified into three groups: a control that received no additional intervention, an MPC group that received an injection of MPCs, and a patch group, which received composite scaffolds containing MPCs in the decellularized scaffold [50]. (H) Schematic of cardiomyocytes (red) in an alginate matrix or in the nanowired composite [84]. (I) Transmission electron microscope images of alginate-nanowire composite scaffolds, where nanowires of approximately 5 μm in length were incorporated into the scaffold material [84]. (J) Connexin-43 (green), troponin (red), and 4′,6-diamidino-2-phenylindole (DAPI) (blue) immunostained cardiomyocytes organized in the nanowired scaffold [84]. (K) Connexin-43 and actinin expression by western blot relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in nanowired group versus the alginate only group 3 or 8 days after seeding with cardiomyocytes. *PE*, Phenylephrine; *SDS*, sodium dodecyl sulfate [84].

ventricle. In addition the cells in the pores were aligned along the preferred direction.

In another study, Feinberg et al. seeded a layer of neonatal rat ventricular CMs on a polydimethylsiloxane membrane that could be detached from a thermosensitive poly(isopropylacrylamide) layer at room temperature, which is called muscular thin films. These cell-covered sheets could be designed to perform tasks such as gripping, pumping, walking, and swimming by careful tailoring of the tissue architecture, thin-film shape, and electrical-pacing protocol [104].

Biophysical cues

Biophysical stimulation has been used to improve the phenotypic maturity of cardiac tissue–engineered constructs. These stimuli are designed to be biomimetic and simulate the native heart environment to facilitate proper cardiac growth and maturation.

Electrical stimulation

One of the primary features of the heart is its electrical connectivity and synchronization. Previous studies have shown improved conductive and functional characteristics of heart tissue grown in the presence of a stimulating electrical field. These results validate the use of electrical stimulation as a functional cue to improve phenotypic maturation of cardiac tissue; however, the precise techniques to apply electrical stimulation to engineered tissues are a topic of further exploration. Various electromechanical cues can be added in order to further recapitulate the cardiogenic niche, allowing true optimization of stem cell derivation procedures [105].

To establish baseline parameters, Tandon et al. compared multiple electrode materials and stimulation regimes to optimize electrical field conditioning of engineered heart tissue [76]. Petri dishes were outfitted with 1/8 in. rod-shaped electrodes, spaced 1 cm apart, and 4 cm in length made from the following materials: carbon, steel, titanium nitride, and titanium. When comparing injected charge and recovery, the electrodes made of carbon recovered the most charge (95%), meaning that they had the least amount of lost charge due reaction products in the solution. The carbon electrodes were also best able to maintain a current through the bioreactor over the 2 ms time range for the depolarizing stimulus. With respect to effect on cell function, the results comparing various electrode materials was more modest, but carbon electrodes still had a favorable trend in their ability to properly capture the pacing of cardiac constructs as well as a lowered excitation threshold. This suggests that the carbon best communicates with the tissue-engineered constructs with minimal side reactions. Based on these studies, the effect of field stimulation improves cardiac tissue function, shown by increased troponin and connexin-43 markers, key proteins in both cell–cell connectivity and contractile apparatus.

Using carbon electrodes in the culture, electrical stimulation has been extensively studied by several research groups to promote functional maturity. Nunes et al. were the first to use a daily step-up protocol to increase stimulation frequency from 1 to 6 Hz within a week. The resulted cardiac tissues showed significant functional improvement in terms of electrophysiology, calcium handling, and cellular morphology compared to nonstimulated controls and 1–3 Hz step-up controls [106]. Ronaldson-Bouchard et al. then changed the step-up stimulation frequency to 0.33 Hz daily from 1 to 6 Hz (Fig. 32.4A) and used cells early after differentiation at day 12. These interventions resulted in a remarkable tissue maturation, including multiple hallmarks of adult myocardium, such as positive force–frequency relationship, adult-like gene expression profiles, remarkably organized ultrastructure (first M line present in engineered cardiac tissues), physiological sarcomere length (2.2 μm) and density of mitochondria (30%), the presence of transverse tubules, oxidative metabolism, and functional calcium handling [86] (Fig. 32.4B–E). Another study done by Zhao et al. had further facilitated chamber-specific stimulation protocols, where ventricular stimulation protocol takes 6 weeks to increase the stimulation frequency from 1 to 6 Hz, whereas atrial protocol involves a 0.4 Hz daily step-up stimulation. The stimulation protocols both resulted in a significant improvement of chamber-specific functions in terms of protein and gene expression, as well as electrophysiology [87].

Mechanical stimulation

Mechanical stimulation approaches the electrical–mechanical coupling of heart tissue from the stretching and mechanical contraction perspective. It has been shown that passive or active tension can increase the cardiac functionality and orientation of cardiac cells. In one study, chitosan, a polysaccharide based scaffolding material, was processed to have defined channel pores for perfusion and high porosity for cell seeding (Fig. 32.4F–H). Due to its attractive mechanical properties (Fig. 32.4I), it was used with a radial stretch device to characterize contraction using active tension. Strain maps of the scaffolds could be generated modeling the surface mechanics of the chitosan, showing stereotyped stress fields around the large pore areas (Fig. 32.4J). Interestingly, increased cell density and organization followed the predicted stress map suggesting that the mechanical stress provides a biophysical cue for cellular organization and communication (Fig. 32.4K) [58].

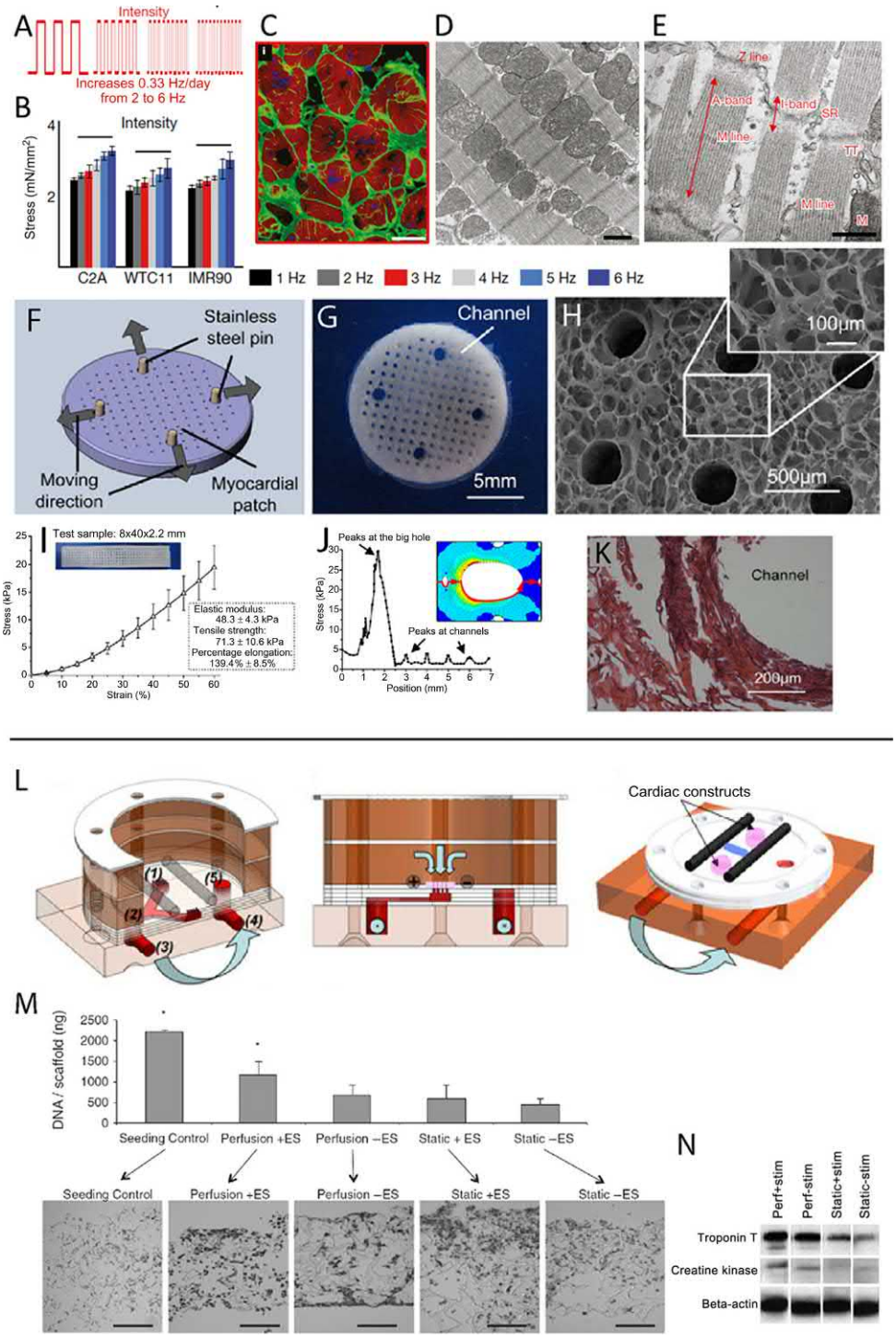

FIGURE 32.4 **Biophysical cues for cardiac tissue engineering.** (A) Electrical stimulation regime increases 0.33 Hz daily from 1 to 6 Hz [86]. (B) The resultant tissue exhibits positive force−frequency relationship [86]. (C) Excitation threshold and TEM of cardiac tissue after electrical stimulation using C2A cell line [86]. (D) Density of mitochondria, scale bars, 1 μm [86]. (E) Sarcomeres, showing A-bands, I-bands, M lines, Z lines, SR and TT. Scale bar, 1 μm [86]. (F) Schematic scaffold design with pins for mechanical stimulation and pores for perfusion [58]. (G) Macroscopic image and (H) scanning electron microscope image of fabricated chitosan−collagen scaffold [58]. (I) Stress−strain curve of scaffold material with corresponding elastic modulus, tensile strength, and percent elongation [58]. (J) Stress distribution along the length of the scaffold [58]. (K) H&E staining of cardiomyocyte laden scaffold in a region near a channel [58]. (L) Schematic of perfusion−electrical stimulation bioreactor [64]. (M) DNA content per scaffold and H&E stained sections of cardiac constructs that underwent different stimulation procedures [64]. (N) Western blot of troponin T, creatine kinase, and beta-actin for constructs that were perfused and/or electrically stimulated [64]. *SR*, Sarcoplasmic reticulum; *TEM*, transmission electron microscopy; *TT*, T-tubules.

Static or cyclic stretching of cardiac tissues often uses specialized mechanical stretcher that applies uniaxial tension to the 3D microtissue constructs. The process mimics the preload of cardiac chamber and aims to improve the electromechanical coupling of the 3D tissue constructs. One study showed evidence that static stretching facilitates better cellular alignment with larger cell area and upregulation of maturation markers in gene expression, including MYH7, TNNT2, NPPA, NPPB, CACNA1C, RYR2, and ATP2A2 [107]. Cyclic stretch was also shown to promote more Cx43 and TNT protein expressions [108].

Moreover, dynamic culture in the combination of increased nutrient availability and constant shear stress stimulation significantly improved the contractile force and conduction velocity of the 3D cardiac patch, both of which have approached the level of adult myocardium [109]. CMs after dynamic culture also have better cellular hypertrophic appearance [109].

Perfusion

Another critical biophysical stimulus—perfusion—is required for proper metabolic function. The transport of fresh oxygen and nutrients along with removal of metabolic products is particularly essential for cardiac tissue. Native tissue combines active perfusion and electrical−mechanical coupling, and in Maidhof et al., these two critical stimuli were combined in a novel bioreactor to enhance cell density and contractile protein expression of troponin [64]. A perfusion system, forcing fluid through a channeled scaffold, was built around the standard paradigm of carbon electrodes (Fig. 32.4L). This resulted in a more uniform cell distribution due to removal of diffusion limitations encountered when growing thicker pieces of cardiac tissue. When electrical stimulation was also applied, the cells were both more numerous while maintain an even density distribution throughout the tissue (Fig. 32.4M). The two stimuli, perfusion and electrical stimulation, had a synergetic effect on troponin expression (Fig. 32.4N). The three biophysical stimuli—electrical stimulation, mechanical stretch, and perfusion—all can contribute to the functional maturity of engineered cardiac tissues.

In vivo applications of cardiac tissue engineering

The ultimate goal of cardiac tissue engineering is the implementation of cells or tissue constructs in an injured heart and subsequently improving cardiac function. An "ideal" cardiac patch would mechanically connect with the host myocardium, integrate with host vasculature, couple electrically with the surrounding myocardium, and generate force in synchrony with the host myocardium to improve the contractile function of a failing heart. We describe in this section two general methods of fabricating a cardiac patch, the first of which focuses on using mechanical load to create force-generating constructs, and the other focuses on introducing multiple cell types to enhance vascularization of the cardiac patch. Open questions for in vivo systems include the functional differences between cell injection and scaffold implantation, and the type of arrhythmogenic or immunogenic response the foreign cells will have when near the host tissue.

Engineered heart issue

Zimmermann et al. outlined an approach to generate contracting rings of cardiac tissue, which can be implanted into infarcted rat hearts, resulting in an improvement in cardiac function [55]. Engineered heart tissues were created by casting neonatal rat CMs, collagen I, and Matrigel in a circular mold, followed by 7 days of culture in 40% oxygen, under static, "auxotonic" load, and in media containing insulin. Five of these loops were then stacked to create a large (15 mm diameter × 1−4 mm thick), fused, synchronously beating tissue assembly amenable to implantation (Fig. 32.5A), similar constructs have been made with hPSC-CMs as well [89,90,110].

Structural and electrical integration with the host myocardium as well as whole heart function were examined in rats with infarcted hearts implanted with engineered heart tissues. Immunostaining of the engineered heart tissue 4 weeks after implantation showed an elongated, sarcomeric pattern suggestive of highly differentiated CMs (Fig. 32.5B). In addition, there was neovascularization within the tissues from the donor cells that connected to the host vasculature (Fig. 32.5C). Electrical coupling was improved in the engineered heart tissues, as demonstrated by lower total activation times and higher QRS amplitudes. In vivo studies allow for the functional examination of whole hearts. Rats with engineered heart tissues demonstrated shorter left ventricular end-diastolic diameter and a lower maximum left ventricular volume when compared to sham-operated rats, suggesting no additional dilation of the infarcted rat hearts as might be expected (Fig. 32.5D). Further, there was no decrease in fractional area shortening of the heart after the operation (Fig. 32.5E).

In another study, Montgomery et al. developed an elastic shape-memory scaffold with a defined microfabricated lattice structure. Because of the unique structure, scaffold seeded with cardiac tissue can be delivered into the heart and other organs through minimally invasive surgery (Fig. 32.5F). Upon delivery the tissue can self-unfold to its original shape without functional losses, and it can be fixed in place with surgical glue or suture. After the delivery the tissue can remain in position for up to 6 weeks. The scaffold also incorporates the center grooves

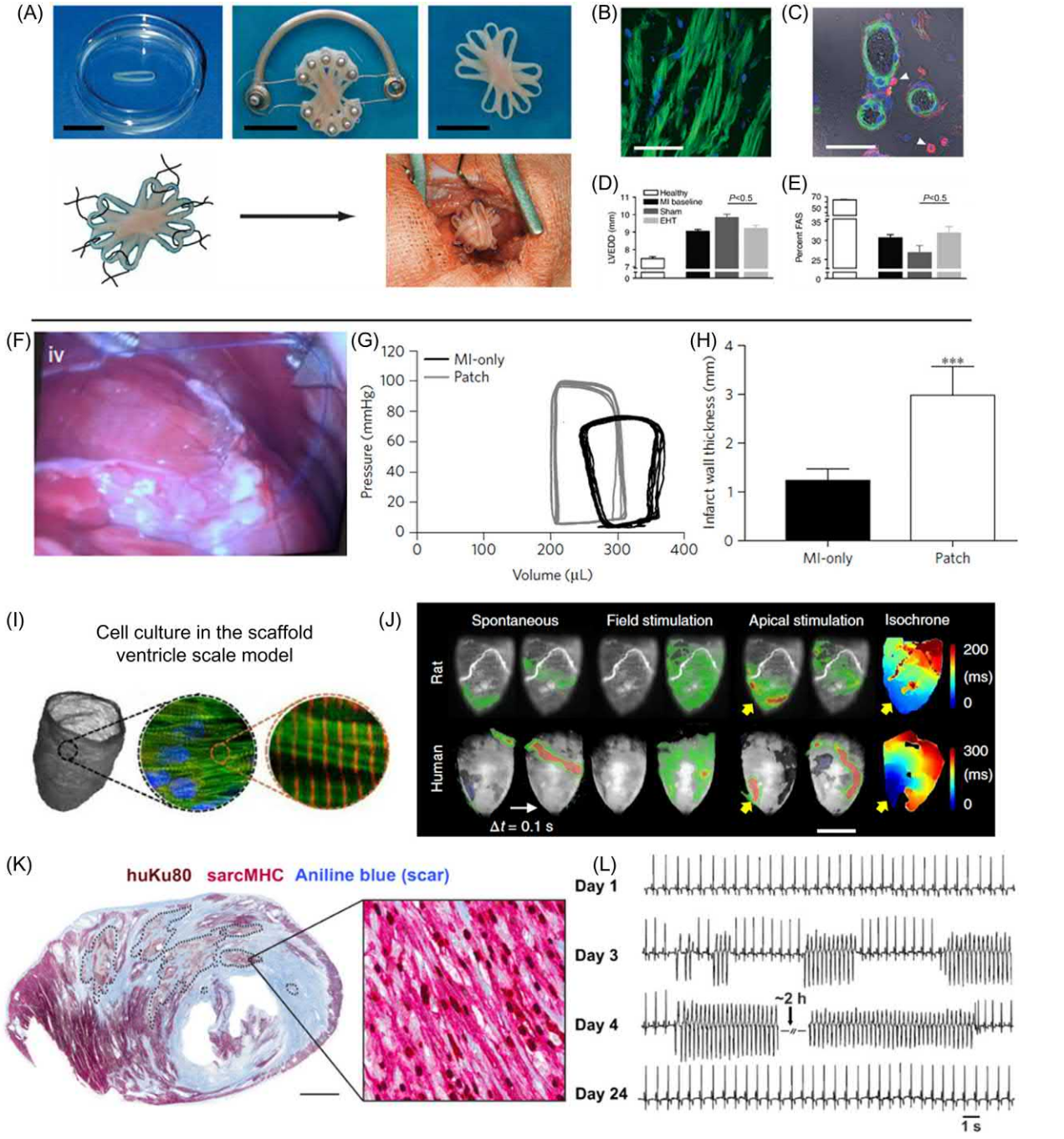

FIGURE 32.5 **In vivo applications of cardiac tissue engineering.** (A) Single ring constructs are stacked in an auxotonic loading system and sewn on a rat heart using six sutures [55]. (B) Troponin (green) and DAPI (blue) staining of engineered heart tissue 4 weeks after implantation showing cardiomyocyte elongation and sarcomeric alignment [55]. (C) Vessel structures where asterisks represent the vessel lumens and arrows represent associated macrophages [55]. (D) Left ventricular end-diastolic diameter and (E) percent fractional area shortening or healthy rat hearts compared to MI baseline, sham surgery, and engineered heart tissue implanted groups [55]. (F) Human embryonic stem cell–derived CM cardiac tissue implantation on the epicardium of the pig heart [88]. (G) Representative PV loops of the rat left ventricle 6 weeks post-MI for MI-only and patch hearts [88]. (H) Representative Masson's trichrome staining of short-axis sections of MI-only and patch hearts 6 weeks post-MI. Scale bars, 4 mm [88]. (I) Circumferentially oriented nanofibers in scale-model ellipsoidal ventricle scaffolds seeded with human cardiomyocytes to produce tissue-engineered ventricles [111]. (J) Calcium transient of the whole tissue–engineered ventricles on day 14. Left: spontaneous activity, middle: field stimulation from far end, and right: apical point stimulation (indicated by *yellow arrows*) with conduction velocities of 9.33 cm/s for Neonatal rat ventricular myocytes (NRVM) ventricles and 5.2 cm/s for hiPSC-CM ventricles [111]. (K) Representative cross section of grafted heart at 14 days posttransplantation showing substantial remuscularization of the infarct scar (blue) by human myocardium (*dotted lines*) [112]. (L) Representative ECG traces acquired on days 1, 3, 4, and 24 posttransplantation suggesting diminished arrhythmias over time [112]. *CM*, Cardiomyocyte; *hiPSC*, human induced pluripotent stem cell; *MI*, myocardial infarction; *PV*, pressure–volume.

for vasculature integration with the host. To evaluate the cardiac patch in vivo, a rat MI model was used to assess the functional effects of cardiac patch implantation. Subcutaneously injected rat cardiac tissues exhibit signs of vascularization and CM persistence. The patch group resulted in smaller scar area and their functional improvement was also evident. The representative pressure–volume (PV) loops measured 5 weeks after patch implantation demonstrated a higher end-systolic pressure and smaller left ventricular volume in the patch group compared with MI-only controls and improved ventricular wall thickness (Fig. 32.5G and H). The delivery method was also validated with porcine model to further conclude the feasibility of the shape-memory tissue patch [88].

MacQueen et al. have recently developed a tissue-engineered model of the whole ventricle [111]. The scaffold was made of nanosized fiber electrospun into a ventricle-like chamber. Because of the architecture of the scaffold, seeded rat CMs or hiPSC-CMs naturally aligned with the fiber orientation, which precisely recapitulated the orientation of native myocardium (Fig. 32.5I). The established tissue-engineered ventricle has a diastolic chamber volume of 500 μL and was capable of ejecting liquid with synchronized contraction. Although the ejection fractions and contractile functions are much smaller than the native rat and human ventricular chamber due to the limited chamber wall thickness (100 μm) and limited cell number, the chamber was well sealed with cells and provided intra ventricular PV measurements and whole ventricle calcium mapping (Fig. 32.5J). By damaging the chamber wall, the engineered chamber can be considered as structural arrhythmia disease model to study the arrhythmia induced by structural damage such as MI through calcium transient mapping [111]. This is the first study that reported the full-scale tissue-engineered heart chamber with human and rat cells, which is the key for drug screening and personalized medicine.

Vascularized cardiac patches

Vascular integration of the cardiac patch with the host myocardium is important for the prolonged survival of thick tissue patches, especially given the high oxygen demand of cardiac tissue. Stevens et al. demonstrated the utility of prevascularizing cardiac tissue prior to transplantation into the infarcted area [48]. Cardiac patches were created from hESC-derived CMs and human umbilical vein ECs, on mouse embryonic feeders. Several experimental groups were investigated: a CM only group, a group consisting of CMs and ECs, and a group consisting of all three cell types. Histology showed that the group consisting of all three cell types resulted in the largest number of vessel-like structures, and the stiffest construct with higher collagen contents, that were most similar to native myocardium. Upon implantation into a skeletal muscle environment, the tri-culture group demonstrated great area staining for β-myosin heavy chain, the greatest number of vessel lumens, and neovascularization and anastomosis with the host vasculature.

Preestablished vasculature in the cardiac patch can significantly promote the integration to the host tissues. A more recent study conducted by Zhang et al. [67] describes a new the organ-on-a-chip model named Angiochip. The Angiochip has multilayered, interconnected, branching lumen that can be coated with ECs and perfused with culture media. The network can significantly improve oxygen and nutrient delivery within the parenchymal tissues by gravity-driven media flow. The smallest microchannel in the network was 100 μm by 50–100 μm, with a wall thickness of 25–50 μm, which is approximately the same size as venules. The Angiochip also has microsized holes on the side of the lumens, which allow on-demand EC sprouting and transmigration into the parenchymal space. The Angiochip is scalable to a thickness comparable to the rat ventricle wall. Because Angiochip is mechanically stable and is capable of withholding blood pressure in rat, the inlet and outlet of the Angiochip can be surgically attached to the femoral vessels on the hindlimbs of adult Lewis rats, in both artery-to-artery and artery-to-vein mode. The two different configurations of direct surgical anastomosis were demonstrated with success, which further demonstrates that the prevascularized cardiac patch can be integrated quickly into the host tissues [67].

Electrical coupling of cardiomyocytes on the heart

One question remains whether or not CMs, when applied to the heart, will form adequate connections with the host tissue, or if the engineered tissue constructs are proarrhythmic, thus potentially negating any therapeutic effect. In one study, Shiba et al. showed that these grafts may electrically couple with the host and suppress arrhythmias [38]. hESC-derived CMs were transplanted onto a guinea pig cryoinjured heart and were subsequently analyzed for electrical coupling with the host tissue. They showed not only 1:1 coupling with the host myocardium, but also the reduced susceptibility of the heart to premature ventricular contractions, and sustained ventricular tachycardia. As a result, this study represents one of the first accounts that CMs are nonarrhythmogenic when implanted. This supports the continued study of the interactions between the native host myocardium and the tissue-engineered construct.

Encouraged by the previous study, Chong et al. had moved to a primate model hoping to better understand

whether clinical-scale hESC-CM transplantation is feasible, safe or can provide sufficient myocardial regeneration in a nonhuman primate model [33]. Due to the large size of injury, one billion hESC-CMs were manufactured and delivered to the injury site. The CM injection generated extensive remuscularization of the infarcted heart with incomplete maturation over a 3-month period. The injected grafts were penetrated with a significant amount of host vasculature. There were also adequate electromechanical junctions between the graft and the host myocytes, which were proved by synchronized calcium transients from the graft and the host electrocardiogram. Despite the success in a small animal, nonfatal ventricular arrhythmias were observed in all hESC-CM-engrafted primates [33].

The injected ESC-CMs typically have a fetal phenotype and were experiencing spontaneous beating. Since guinea pigs and other small animals have a much faster heart rate than humans, it is possible that the spontaneous beating from the human CMs was proarrhythmic but was covered by the fast heart rate in small animals. When the host heart rate is low, the higher spontaneous beating rate would manifest. In a recent publication using a porcine model [112], similar remuscularization and functional improvements were reported to occur after hESC-CMs transplantation into hearts (Fig. 32.5K). Although monomorphic ventricular tachycardia was routinely found in grafted animals, the arrhythmia eventually disappeared potentially due to the maturation of grafts over time (Fig. 32.5L).

In comparison with single cells, engineered cardiac tissues have been shown to exhibit much lower spontaneous beating rate after maturation regimes [86,87], which may minimize or eliminate the risk of arrhythmias upon implantation.

Weinberger et al. implanted engineered cardiac tissue made of hiPSC-derived CMs and hiPSC-derived ECs [113]. The grafts were transplanted onto large defects (22% of the left ventricular wall, 35% decline in left ventricular function) of guinea pig hearts 7 days after cryoinjury. Twenty-eight days after transplantation, the hearts repaired with tissue that had remuscularized 12% of the infarct area. These grafts showed CM proliferation and vascularization; however, the evidence for electrical coupling to the intact heart tissue was limited. Another similar study demonstrated that implantation of engineered cardiac tissues did not provoke or attenuate ventricular arrhythmias [114]. It is possible that due to the extensive level of cellular reorganization during tissue compaction phase of engineered cardiac tissues, less cell−cell interaction would occur during the implantation to facilitate electromechanical integration. Therefore limited success was achieved for engineered cardiac tissue implantation.

Modeling of disease

In vitro models of disease represent an important avenue in studying disease and identifying potential therapeutic options. Here we describe the use of patient-specific cells to generate diseased CMs. We further describe the utility of tissue engineering in providing a faithful representation of diseased myocardium, and how this may be used in high-throughput screens and drug studies.

Generation of patient-specific cardiomyocytes

iPSCs have allowed for the generation of patient-specific CMs. One of the first accounts came from Moretti et al., who generated iPSCs from patients with a particular genetic mutation leading to long QT syndrome and subsequently differentiated them into CMs (Fig. 32.6A and B) [79]. Localization of the mutated channel (KCNQ1) in the endoplasmic reticulum confirms this channel as the likely cause of disease (Fig. 32.6C and D). These CMs displayed the electrophysiological phenotype of long QT syndrome, including the classic lengthened action potential duration (Fig. 32.6E and F). The use of induced pluripotent cells to recapitulate disease phenotype is particularly amenable to patients with genetic mutations. Since the studies on long QT syndrome, CMs from patients with other diseases, including arrhythmogenic right ventricular cardiomyopathy and familial dilated cardiomyopathy (DCM), have been generated [80,115]. The iPSCs from patients harboring genetic cardiac mutations have been differentiated into CMs. These include cells from Timothy [116], long QT [79], and LEOPARD [117] syndromes and DCM patients [80].

Engineered heart tissue models

While induced pluripotent cells are useful in generating cells from patients with specific genetic mutations, many cardiac diseases are multifactorial and require a biomimetic environment to faithfully reproduce the disease phenotype.

Cardiac fibrosis

Engineered cardiac tissue can be used to model myocardial fibrosis, which is a severe global health problem due to its prevalence in all forms of cardiac diseases. Wang et al. [118] have demonstrated an in vitro disease model of human myocardial fibrosis. The disease model is constructed with an excessive initial cardiac FBs population. The resultant tissue has massive collagen production and sparse distribution of CMs, which precisely recapitulate contractile, biomechanical, and electrophysiological complexities of fibrotic myocardium (Fig. 32.6G and H). By

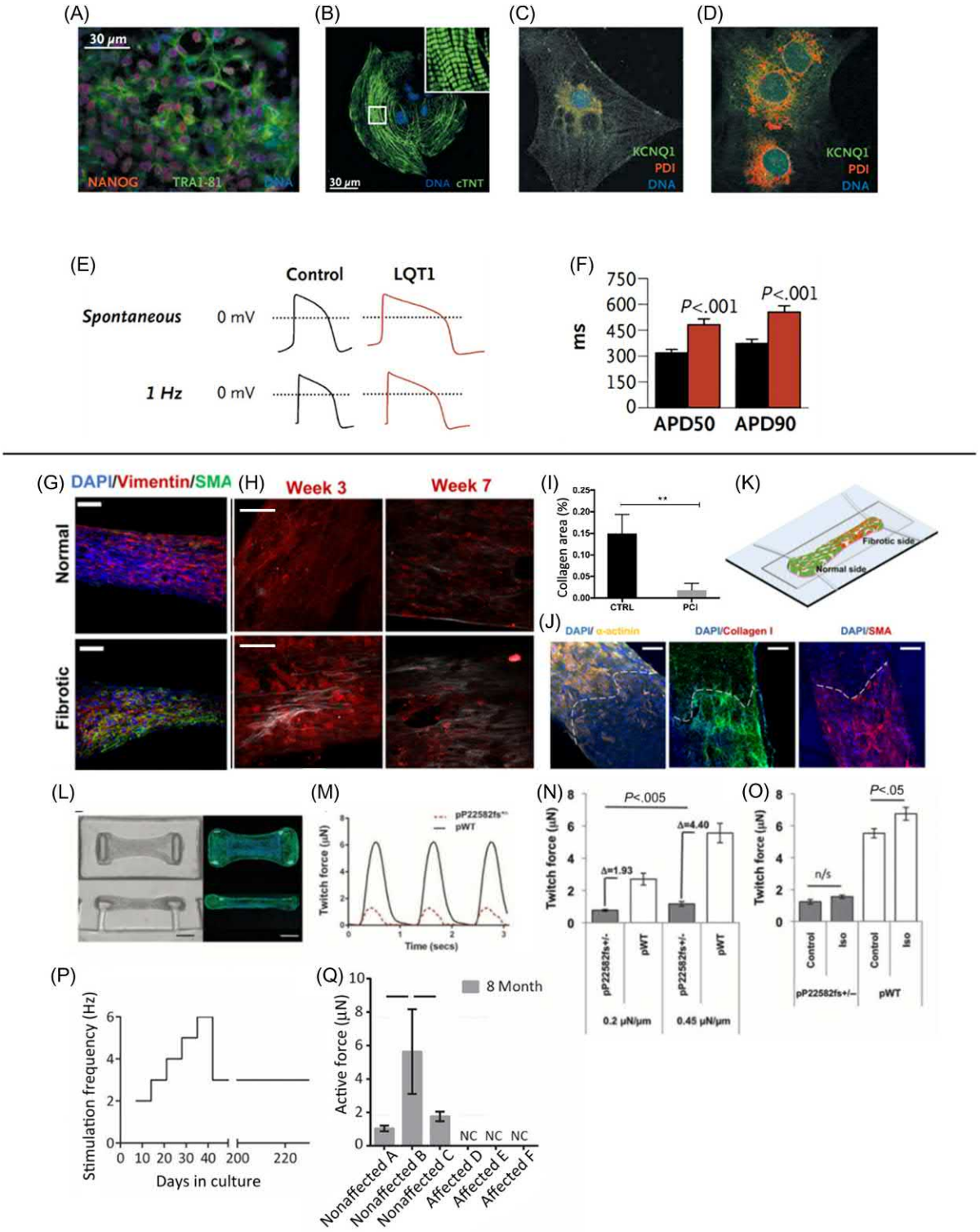

FIGURE 32.6 **In vitro models of disease.** (A) immunostain of induced pluripotent cells from LQT1 patients for Nanog and Tra1-81 [79]. (B) Troponin staining showing sarcomeric organization in iPS-derived cardiomyocytes. KCNQ1 (green) and Protein Disulfide Isomerase (PDI) (red) staining for control cardiomyocytes (C) and patient cardiomyocytes (D) [79]. (E) Single-cell action potentials of control and patient-derived stem cells spontaneously beating or stimulated at 1 Hz [79]. (F) APD50 and APD90 for control (black) or patient-derived (red) cardiomyocytes [79]. (G) Immunostaining images of normal and fibrotic tissues double-stained for vimentin and α-SMA (scale bar = 100 μm) [118]. (H) SHG images to reveal collagen content in normal and fibrotic tissues at week 3 and 7 of cultivation, (scale bar = 100 μm) [118]. (I) Schematics of the integrated scar-myocardium model [118]. (J) Representative immunostaining images of the integrated tissue stained for sarcomeric α-actinin, collagen type I, and α-SMA. (scale bar = 100 μm) [118]. (K) Quantification of fibrotic tissues treated with antifibrotic compound for 7 days (mean ± SD, $n \geq 3$, Student's *t*-test) [118]. Comparison between engineered cardiac tissue−derived from wild type (pWT) and titin truncated diseased cell line in terms of (L) bright field and fluorescent image (scale bar = 50 mm), (M) force traces, (N) active force responses to different pillar stiffness, and responses to isoproterenol [119]. (P) Electrical stimulation regime simulating chronic cardiac overload to induce disease phenotype [87]. (Q) Absence of contractile forces in the affected groups in comparison to the nonaffected groups [87]. *iPS*, Induced pluripotent stem; *SHG*, secondary harmonic generation.

using this disease model, evaluation of antifibrotic compounds can be carried out with human models with clinical relevance (Fig. 32.6I). Taking a step further, a heteropolar integrated model was constructed with a fibrotic end and a healthy end, which may capture the regional heterogeneity of scar lesion, border zone, and adjacent healthy myocardium [118] (Fig. 32.6K and J).

Titin mutation–related dilated cardiomyopathy

Engineered cardiac tissue can also be used to model DCM, which is mostly caused by a genetic mutation of sarcomere protein titin (TTN-truncating variants). By using patient-specific cell lines, engineered cardiac tissue can be a powerful tool to evaluate the pathogenicity of titin gene variants. To be more specific, the model recapitulated the A-band domain of TTN mutation caused DCM in terms of sarcomere insufficiency, impaired responses to mechanical and β-adrenergic stimulation, and attenuated growth factor and cell signaling activation. The titin truncation caused the missing link between sarcomerogenesis and adaptive remodeling [119] (Fig. 32.6L–O).

Diabetes-related cardiomyopathy

Engineered heart tissue has been used by Song et al. to study diabetes-related cardiomyopathy and the effect of drug therapy on diabetic engineered heart tissues [120]. Briefly, the diabetic rat heart treated with high concentration of glucose exhibited diminishing electrophysiological properties and increased ratio of myosin heavy chain isoform β to α, both of which were indications of diseased states of the heart.

Chronic hypertension induced left ventricle hypertrophy

Chronic hypertension is a well-known risk factor for left ventricular hypertrophy. To model this polygenic disease, cardiac tissues were made with six patient–specific cell lines from hypertensive patients, only three of which experienced left ventricle hypertrophy. Zhao et al. designed an 8-month long electrical stimulation protocol to first serve as a maturation protocol and then chronic cardiac overload to simulate hypertension (Fig. 32.6P). After the protocol, only the tissues from patients with left ventricle hypertrophy had completely seized contraction, while others retained some level of contraction with electrical pacing (Fig. 32.6Q). The tissue model with specialized electrical conditioning successfully manifests the disease phenotypes, in terms of function and genetic profiles. Many polygenic diseases only display disease phenotypes after reaching adulthood, which requires significant CM maturation in vitro [87].

Barth syndrome

Barth syndrome is a mitochondrial disorder caused by a genetic mutation of the Tafazzin, which can lead to mitochondrial cardiomyopathy. Using patient-specific CMs, disease phenotypes manifested through metabolic, structural, and functional abnormalities. To be more specific, the diseased tissue models have smaller mitochondria, much lower metabolic activity, and abnormal sarcomerogenesis, which has been fully resolved in isogenic cell line–derived tissues. Therefore the study successfully recapitulates Barth syndrome with an in vitro tissue model to study the disease mechanisms [121].

Tissue engineering as a platform for pharmacologic studies

Given the active nature of cardiac tissue, new methods have been developed to study the function of engineered cardiac tissues in multiplexed in vitro systems [122]. Particularly, Schaaf et al. cast hESC-derived CMs in fibrin across a 24-well format that allowed the real-time measurement of force generation [123]. This system was then used to examine the effect of various proarrhythmic drugs (e.g., E-4031, quinidine, procainamide) on the beating dynamics of the heart tissue. As expected from the known electrophysiological effects of the drugs, the engineered tissues displayed irregular beating at low relaxation velocities.

In a similar fashion the Biowire platform also enables cultivation of miniaturized, high fidelity cardiac tissues and high-content online functional readouts to facilitate drug screening and toxicity evaluation [87]. In addition to commonly tested proarrhythmic drugs (e.g., E-4031) and currently used therapeutic drugs (isoproterenol, lidocaine, milrinone, diltiazem, nifedipine, and thapsigargin), Wang et al. used their preestablished fibrotic tissue model to screen a group of small molecule candidates for their antifibrotic potential. The results had preliminary success in that one small molecule candidate exhibited a significant antifibrotic tendency and significantly reduced the collagen content in the fibrotic cardiac tissue [119]. In another study, cardiac tissue model of Barth syndrome was also used to find a therapeutic target. By assessing ATP levels and mitochondrial function, linoleic acid would be the ideal candidate to correct the metabolic phenotype of Barth syndrome iPSC-CMs [121].

In a separate study, sheets of engineered cardiac tissues were fabricated in a similarly multiplexed format, which allowed the measurement of stress exerted by the cells [124]. Myocytes were seeded on a micropatterned surface to facilitate alignment of CMs. The system was imaging compatible, allowing for the quantification of

images or videos to determine contractility, action potential propagation, and cytoskeletal architecture.

Summary and challenges

When considering all the challenges facing the field and the methods which individual groups use to find answers, it becomes obvious that the biggest obstacle that must be overcome is better standardization of experimental and measurement techniques as well as properly defining the level of cardiac regeneration after insult (functional, morphological, etc.) deemed to be successful. The reasons for this are clear when one considers the results reported by different groups. While it seems that all of the in vivo models discussed are similar, rarely can a direct comparison between results be done. All discussed studies report significant improvement in function; however, looking at ejection fraction alone groups reported anything from ~12% to 77% difference between the treatment group and MI-only as being significant and clinically relevant [33,113,114,125–129]. One could argue that the greatest improvement corresponds to the best system. However, when one examines different models discrepancies regarding timing of application of cells and tissues, different animal models and evaluation become obvious.

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Chapter 33

Blood vessels

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Introduction

In the early 1900s, Alexis Carrel first described the utility and limitations of autogenous and synthetic grafts, including the unique challenges of small-diameter synthetic grafts. Fifty years later, Voorhees et al. first utilized a vascular graft for larger artery replacement [1]. In 2019 small-diameter synthetic grafts continue to have worsened long-term patency rates compared to autogenous vessels.

Since patients with cardiovascular disease are living longer, there is a growing need for more vascular interventions. Annually, there are between 600,000 and 1.4 million bypass operations in the United States, and this number has remained stable in recent years. There has also been a significant growth in endovascular procedures (e.g., angioplasty and stenting) for the lower extremity, together these efforts have correlated with a decrease in major amputations [2]. Despite periprocedural benefits of endovascular approaches (decreased early mortality and morbidity), endovascular procedures have inferior durability. Thus there remains a critical need to create small vessel vascular conduits and to apply bioengineering approaches to endovascular therapies to improve the effectiveness of all vascular interventions for the treatment of cardiovascular disease and injury in patients.

This chapter discusses the native vasculature, clinically available and experimental bypass grafts, including tissue-engineered vascular grafts (TEVG), and modifications of these grafts to facilitate healing, function, and efficacy.

Normal and pathologic composition of the vessel wall

For arteries, the intima (tunica intima) is composed of a relatively quiescent endothelial cell (EC) monolayer and its surrounding basement membrane proteins (e.g., type IV collagen, perlecan). Together with the underlying internal elastic lamina, the intima maintains vascular smooth muscle cells (VSMCs) in their contractile state and inhibits pathologic VSMC activity. Deep to the intima and separated by the internal elastic lamina is the medial layer (tunica media). It is the thickest arterial layer, and in nonpathologic states it is composed of VSMCs and many extracellular matrix (ECM) proteins (e.g., elastin and the fibrillar collagens such as type I collagen). Medial VSMCs here respond to intimal cues to dilate or contract the vessel. The medial layer in veins is difficult to define because it lacks an internal elastic lamina and has limited VSMCs, but after exposure to arterial flow via vein graft bypass, there is robust proliferation of VSMCs in the media of veins that approximates that of native arteries [3]. The external elastic lamina defines the abluminal edge of the media, and the vaso vasorum is prominent in the outer adventitial vessel layer (tunica adventitia). This vaso vasorum provides for the metabolic needs of approximately the outer two-thirds of the vessel wall. The adventitia is composed of loosely arranged connective tissue and fibroblasts, along with some progenitor cells [4], and it may play an important role in the progression of restenosis and late interventional failure after angioplasty. This is inferred from more favorable vessel remodeling after adventitial delivery of therapeutics [5].

In the absence of disease or injury, native blood vessels possess an endothelial lining that constantly secretes bioactive substances promoting fibrinolysis and inhibiting thrombosis and VSMCs switching from a contractile to synthetic phenotype [6]. In addition, the artery is more than a pipe. It is a complex tissue that responds (radially and longitudinally) to the three components of the cardiac cycle to accommodate the systolic flow bolus of blood,

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provide resistance to maintain blood pressure during early diastole, and then propulse blood distally during late diastole. This latter phase is the only part of arterial flow dependent on the artery rather than the heart and is critical to maintaining communication between the heart and the tissue beds.

Developmental biology cues important in vascular tissue engineering

During development artery and venous identity is quickly decided by a complex and highly orders series of signaling and mechanical stress-mediated molecular pathways [7]. Once determined, there are obvious and lasting matricellular differences between arteries and veins that are important to this chapter's focus on blood vessels and blood vessel replacement strategies.

Key among these differences is the completeness of the medial layer and the mechanical differences due to the amount and orientation of elastin [8]. The undisturbed vein has vastly different solid mechanics than the artery, and the adventitial collagen is the key component of vein mechanics. This collagen enables veins to withstand great pressures (such as those generated by standing) [9]. However, the pulsatility or hysteresis of veins is lost at ~25 mmHg, and veins require skeletal muscles to extrinsically push the blood from the lower extremities toward the heart. In contrast, healthy arteries keep pulsatility at arterial pressures. This is due in large part to Windkessel effect, which is functionally derived by the elastin content and orientation [10].

Unfortunately as we age, both arteries and veins do not deposit more elastin. In fact, elastin degradation is the rule with aging [11,12]. With aging, vessels do develop more collagen, creating a stiffened phenotype that impacts cell biology and perfusion of target tissues [13,14].

Vessels, particularly arteries, are known to contain and attract progenitor and immune cells in their walls. These cells interact with the more prominent endothelial (intima), VSMCs (media), and fibroblasts (adventitia) to mediate homeostatic changes in the vessel wall thickness and remodeling cues, including axial and radial growth or shortening [15,16].

Conduits

Arteries

Few autogenous arteries are available for use as conduits due to the lack of redundant arteries in the body, and the lengths of available arteries limit their use to the short bypasses in the coronary circulation. In cardiac surgery, the use of the internal mammary artery has outstanding long-term patency rates that far exceed that of vein grafts [17]. The benefit is less clear when different arteries (e.g., radial artery) are used for coronary bypass grafting harvesting [18]. The theoretic advantage of using arteries over veins is that arteries have already been functioning under arterial flow conditions, whereas veins need to "arterialize" after exposure to arterial flow. However, all bypass targets are not identical, and graft remodeling is also determined in part by the hemodynamics of the perfusion bed as demonstrated with vein grafts to the myocardium of the heart compared to the skeletal muscle of the lower extremity in nonhuman primates [19].

Veins

Superficial and deep veins can be utilized as conduits for arterial bypass, but superficial veins are better tolerated and more commonly used. The benefit of veins over synthetic grafts for small-diameter graft bypasses is well known for most bypasses [20]. The greater saphenous vein is the most commonly used conduit for coronary and infrainguinal bypass grafting, and it is duplicated in ~8% of persons [21]. The lesser saphenous vein is shorter in length and not easily accessible in the supine position, but it can be useful as a conduit when working posteriorly or through a separate harvest incision [22–24]. Cephalic veins can also be used but due to their decreased length, they are often spliced together for long bypasses after a bilateral arm vein harvest [25,26].

Once implanted, veins arterialize, and the ECs in the vein appear to lose their venous identity but they do not gain arterial identity [27], which may contribute to vein graft failure rates. Since vein graft arterialization stimulates desirable and undesirable biologic responses, it is not surprising that a number of vein grafts fail or require adjunctive procedures to maintain patency (Fig. 33.1). The mode of failure is more prominent at certain times after implantation. Initially, vein graft failure is usually technical, that is, related to the technical aspects of the implant procedure. Between 2 months and 2 years, failure usually is from myointimal hyperplasia (frequently at anastomotic or valve sites), and after 2 years, it is most commonly due to progression of atherosclerosis in the inflow and/or outflow arteries, or in the vein graft proper.

Current status of grafts in patients

Conduit patency and failure

The results of the PREVENT III and IV trials, which tried to arrest vein graft proliferation, have demonstrated that vein graft failure is relatively common and similar after both infrainguinal and coronary revascularizations [28,29]. And the primary patency rates for infrainguinal bypass are strikingly similar to those reported more than

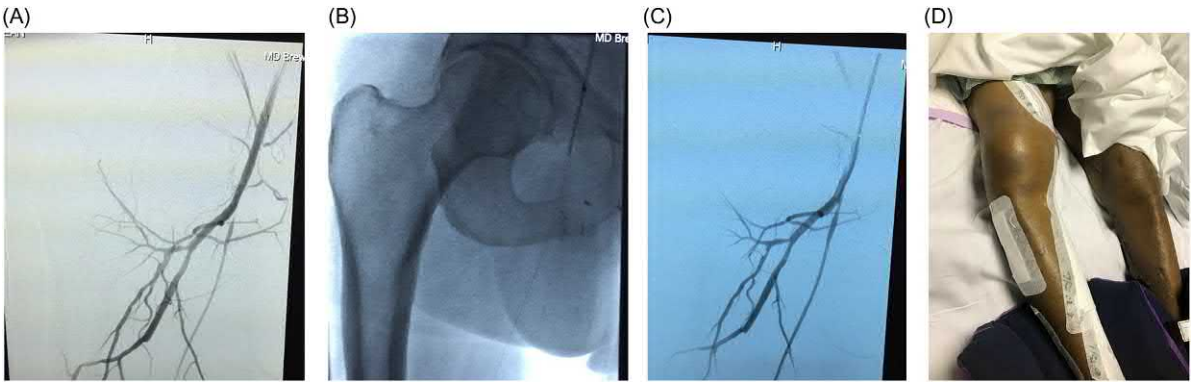

FIGURE 33.1 Vascular interventions. (A) Vein graft stenosis (*denoted by arrow*) just distal to the proximal anastomosis. (B) Angioplasty balloon delivery to area of stenosis. (C) Satisfactory resolution of vein graft stenosis post angioplasty. (D) Bandages mark incisions for saphenous vein harvest and use in a femoral to anterior tibial artery bypass in a patient with severe PAD. *PAD*, Peripheral arterial disease.

30 years ago [20]. However secondary patency rates are better, emphasizing the importance of vein graft surveillance.

A variety of mechanisms can also lead to synthetic vascular graft occlusion. Immediate graft failure is usually the result of technical error from the operation or the patient having a hypercoagulable status; failure in the first month following graft placement is most likely the result of thrombosis secondary to poor distal blood flow. Smaller-diameter grafts have lower flow velocities that are thought to promote thrombosis and make them particularly susceptible to anastomotic myointimal hyperplasia (IH). Thus graft thrombogenicity is an enduring concern for vascular grafts. Anastomotic pseudointimal hyperplasia is the most common reason for graft failure from 6 months to 3 years after graft insertion, and later graft failure is frequent due to the progression of distal atherosclerotic disease. Pathophysiologically, injured intima or exposed luminal area of a graft may lead to thrombosis via platelet deposition and activation of the coagulation cascade, and over time it promotes pathologic smooth muscle cell (SMC) migration, proliferation, and ECM deposition, leading to IH. IH in turn narrows the vessel lumen (restenosis) disturbing blood flow to the point that it may occlude, thrombose, or cause symptomatic ischemia in the relevant distal end organs such as the brain (stroke), heart (myocardial infarctions), and extremities (acute or critical limb ischemia).

Venous reconstruction

Ring-reinforced expanded polytetrafluoroethylene (ePTFE) grafts are commonly used for large-caliber venous replacements in unusual circumstances, such as the replacement or bypass of the inferior or superior vena cava [30–33], iliofemoral, jugular [34], and portal vein [35] or the construction of portosystemic shunts for portal hypertension [36,37]. The ring-reinforcement in theory resists respiratory compression better and thus prevents graft collapse that may be a factor in promotion of thrombosis. However, no comparative study with nonringed grafts has been done and is not feasible given the small numbers of patients in even the largest reports with these procedures. A temporary arterial graft to these venous reconstructions may improve their patency [38].

Breuer and Shinoka have now implanted their bioengineered vena cava replacement into 25 patients. With an average follow-up of 11 years, they have had no graft-related mortality, and the seven stenotic grafts identified were all successfully treated with balloon angioplasty [39]. This exciting success in children may have broadening impact on developing bioengineered grafts for clinical use in other anatomic locations.

Hemodialysis vascular access

Typically, long-term access to the vascular system for hemodialysis is provided by an arteriovenous (AV) fistula or an AV graft. Synthetic AV grafts account for approximately half of all permanent grafts placed in patients with incident end-stage renal disease in the United States. However, only 26% of them remain patent without complication 2 years after placement [40]. The secondary 3-year patency is better at 42%–60% [41–43]. The failure of synthetic AV grafts is often associated with stenosis at the venous outflow anastomosis, which leads to subsequent thrombosis. Again, infection is the most dreaded risk of synthetic graft use for hemodialysis. This population is particularly susceptible to infection because renal failure suppresses the immune system, and there is a potential for graft contamination at each dialysis session.

ePTFE grafts are usually the synthetic choice for dialysis access when a primary AV fistula for hemodialysis cannot be performed or has failed. Dacron grafts often

have patency, bleeding, and wall integrity difficulties. Various modified grafts have been studied for many years in an attempt to improve the performance of the synthetic grafts for the angioaccess application. The modifications to synthetic materials have included changing the wall structure, adding luminal or extramural coatings, and incorporating additional layers to limit postdialysis bleeding. ePTFE can be modified into a stretch, thin, or thickened wall configuration that certainly affects handling but may also impact graft function over time [44].

Dialysis access grafts procedures require a period of time after implantation prior to use. Autogenous AV fistulas typically require a maturation period of 6 weeks to 2 months before first cannulation, whereas synthetic grafts are typically delayed 2–4 weeks prior to access to permit incorporation of the graft into the surrounding tissue. There are a couple of grafts on the market that are designed for earlier access. The Flixene, Avflo, Acuseal, and Vectra are examples. Primary patency rates for these grafts are generally poor but secondary patency rates are acceptable [45]. The handling of these grafts is also technically more challenging as they are more rigid than standard hemodialysis grafts [46].

Inflammation and the host response to interventions and grafts

The inflammatory response to vascular interventions is complex because these patients have systemic inflammation, which may influence the degree and direction of local inflammation. Potent chemoattractants such as complement 5a (C5a) and leukotriene B_4 recruit neutrophils to the graft surface where they localize in the fibrin coagulum of the graft's inner and outer capsule via β2 integrins. Also, IgG binds to the neutrophils' Fcγ receptors activating neutrophils' proinflammatory response while inhibiting normal clearance of bacteria. Control of neutrophil response (and Netosis) may have great benefits in vascular tissue engineering applications [47]. Neutrophils also interact with various other deposited proteins, including C3bi and factor X, and they adhere to the ECs in the perianastomotic region through selectin- and integrin-mediated mechanisms. L-selectin is thought to modulate neutrophil/EC interactions by presenting neutrophil ligands to both E- and P-selectin on the vascular endothelium. In addition, selectin–carbohydrate bonds are important for the initial cellular contact while the integrin–peptide bonds are responsible for strengthening this adhesion, as well as the transmigration of neutrophils. Both intercellular adhesion molecular-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the EC surface bind these integrins as well, and ECs upregulate ICAM-1 and express VCAM-1 when stimulated by inflammatory agonists such as interleukin (IL)-1, tumor necrosis factor (TNF), lipopolysaccharide, and thrombin. Further, activated neutrophils release oxygen-free radicals and various proteases, which result in matrix degradation and may inhibit both endothelialization and tissue incorporation of the vascular tissue and grafts [48].

Circulating monocytes/macrophages are also attracted to areas of injured or regenerating endothelium, especially in response to IL-1 and TNF-α. There are many plasma monocyte recruitment and activating factors, including LTB_4, platelet factor 4, and platelet-derived growth factor (PDGF). This process is propagated in the presence of these plasma activating factors, driving monocytes to differentiate into macrophages that direct the host's chronic inflammatory response via the release of proteases and oxygen-free radicals. They also are thought to be critical to promoting arteriogenesis via monocyte chemotractant protein-1.

A variety of cytokines are released from the inflammatory cells activated by vascular grafts. Lactide/glycolide grafts are composed of bioresorbable materials that are phagocytosed by macrophages; in culture these materials stimulate macrophages to release mitogens that stimulate vascular cells. This mitogenic activity appears to be related to the secretion of fibroblast growth factor (FGF)-2 since pretreatment of the culture media with a neutralizing anti-FGF-2 antibody significantly diminishes the stimulatory effect on SMC growth in culture [49]. Cultured monocytes and macrophages incubated with Dacron and ePTFE have been demonstrated to produce different amounts of IL-1β, IL-6, and TNF-α that are biomaterial specific [50]. TNF-α is one of the factors that may contribute to the enhanced proliferation of SMCs caused by leukocyte-biomaterial interactions, while IL-1 may be partly responsible for the increased SMC proliferation caused by leukocyte-EC reaction. IL-1 also induces upregulation of insulin-like growth factor-1 expression in ECs, and coculture of neutrophils with IL-1β-treated ECs dramatically increases PDGF release.

It is attractive to think that the host inflammatory response can be manipulated to promote favorable cellular and protein responses with the goal of promoting autogenous ingrowth of TEVGs. Recently, Shinoka and Breuer have demonstrated that the populating cells can be manipulated by modifying the homing cells with angiogotensin inhibitors [51]. Since the inflammatory reaction elicits a cascade of growth processes, it has also been proposed that approaches attenuating the initial inflammatory reaction may improve long-term graft patency. Over time certain inflammatory and profibrotic responses limit the long-term patency of vein grafts [52–54] and can promote a more aggressive atherosclerosis via endothelial to mesenchymal transition [55]. Excitingly focused antiinflammatory interventions may be able to counter this

inflammatory process [56]. Similar inflammatory processes are likely involved in arterial disease. We have recently demonstrated that flow-mediated arterial stiffening occurs through specific and reversible profibrotic pathways in both mice and patients with peripheral arterial disease (PAD) [57], and now for the first time, investigators have demonstrated that antiinflammatory medication can decrease cardiovascular events such as strokes and heart attacks [58]. These pathways are likely also important to AV fistula and biologic graft function [51,59], and clinically available blockade of angiotensin signaling pathways may limit this process [51].

Host environment and the critical role of the endothelium

It is well known that smoking, diabetes, renal disease, and atherosclerosis all have a significant effect on blood vessels (Fig. 33.2), so it is not surprising that these conditions also affect bioengineered vessel remodeling [60,61] through aneurysm [62,63] and calcification [64,65]. Unfortunately, the lack of clinically relevant and aged animal models has limited solution-based testing of TEVG in these specific diseases.

Arteriogenesis or the growth of existing collateral arteries is one promising way of improving perfusion in persons with obstructive vascular disease. Newer models of arteriogenesis may be useful in translating effective strategies of this approach [66]. However, the more clinically relevant the animal models are, the less arteriogenic capacity exists [67].

Since thrombogenicity and intimal hyperplasia represent the most common causes of graft failure and are both mediated at the luminal interface of the vessel or graft, the inner lining of grafts has been the subject of much investigation. Unfortunately, and unlike most animal models in use that spontaneously endothelialize synthetic grafts, humans manifest only limited EC ingrowth not extending beyond 1−2 cm of both anastomoses. However, endothelial islands have been described in the midportions of grafts at significant distances from the anastomosis, suggesting that other EC sources for graft endothelialization may exist. Interstitial tissue ingrowth accompanied by microvessels from the perigraft tissue is one potential source. There is also evidence that circulating ECs, endothelial progenitor cells (EPCs), or stem cells can be directed to these areas. Such homing can be promoted through affixation of EC attractant antibodies to the grafts in a similar fashion to that utilized in coronary artery stents [68].

However, ECs growing on synthetic graft surfaces are not necessarily the same as their normal quiescent counterparts in uninjured vessels. These ECs are often "activated," secreting bioactive substances (e.g., PDGF) that actually promote thrombogenesis and changes in SMC phenotype. This has been seen in the perianastomotic region, which is the most frequent site of interventional failure after implantation. SMCs found within the myointima of synthetic grafts are also functionally altered. They produce significantly higher amounts of PDGF, as well as various ECM proteins, compared to those of the adjacent vessel, which along with the body's inflammatory reaction to synthetic material, contribute to the development of intimal hyperplasia [69]. Incomplete luminal surface cellular coverage can be found at the mid-region of synthetic grafts, even years after implantation [70,71].

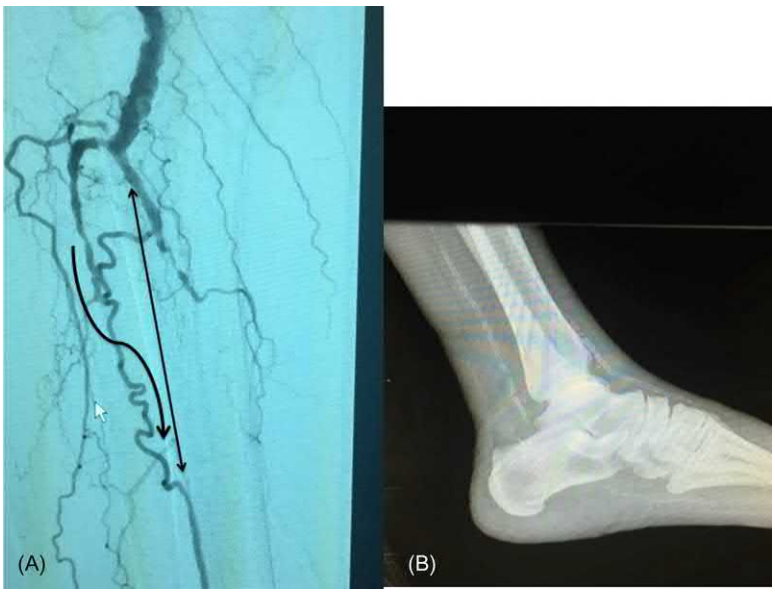

FIGURE 33.2 Vascular pathology in PAD. (A) Severe obstructive PAD in a diabetic patient. Tibial arteries are all occluded proximally. Arteriogenic (*curved arrow*) collaterals have developed to link proximal blood flow to distal peroneal artery (*straight arrow*). (B) Calcified arteries are evident in plain X-ray of the foot in an end stage renal disease patient. *PAD*, Peripheral arterial disease.

Prevalent grafts in clinical use

Cryovessels

Cryopreserved allografts provide an alternative to femoral vein harvest or antibiotic soaked Dacron with omental flap coverage for infected aortoiliac reconstruction [72]. They have also been reported to provide acceptable limb salvage rates. However, their exceptionally poor primary patency rates for infrainguinal bypass [73–75], even with immunosuppressive therapy [76], and tendency toward aneurysmal dilation and blowout limit the use of these conduits to patients without leg or arm vein and the presence of infection.

Synthetic grafts

Readily available large diameter synthetic grafts exposed to high flow conditions, such as those used for aortic reconstruction, have a superb 5-year patency rates (~80%) [77]. Conversely, when small-diameter synthetic grafts are used in lower flow environments such as the infrapopliteal region, the results are poor with 1- and 3-year patency rates for of 43% and 30%, respectively [20]. Since we cannot change to flow environments of different arterial beds, work has been done to better match the graft to the vessel size and flow environment.

Polyethylene terephthalate (PET or Dacron) and ePTFE are the predominant materials currently used in synthetic vascular grafts. Both Dacron and ePTFE react with blood components and perigraft tissues in clinically desirable and undesirable manners. In fact, all grafts, regardless of their composition and structure, evoke complex but predictable host responses that begin immediately upon restoration of perfusion.

Dacron

Dacron was first introduced in 1939, and it was patented by DuPont as Dacron fiber in 1950. Vascular grafts made from Dacron were adopted early by Julian and DeBakey [78,79]. Clinically available Dacron grafts are fabricated in either woven or knitted forms. In woven grafts, the multifilament Dacron threads are fabricated in an over-and-under pattern in both lengthwise (warp) and circumferential (weft) directions. This structure results in limited porosity but the best dimensional stability of the finished grafts. Accordingly, woven grafts have less bleeding through interstices and less likelihood of structural deformation after implantation. Knitted grafts employ a textile technique in which the Dacron threads are looped to form a continuous interlocking chain. Crimping technique is utilized to increase flexibility, distension, and kink resistance of textile grafts. Crimping can decrease thrombogenecity of vascular grafts [80]. Crimping may also reduce the effective internal diameter of the graft and creates an uneven luminal surface. The latter can potentially interfere with laminar blood flow that leads to increased thrombogenicity of the graft. While such considerations may not be critical in large-diameter grafts, it is very important in small-diameter situations. As a result, synthetic rings or coils are applied to the external surface of grafts to provide external support to resist kinking and mechanical compression.

Dacron grafts, especially knitted grafts, are prone to dilate when implanted into the arterial environment. A 10%–20% increase in graft size upon restoration of blood flow is considered to be in expected range. Direct relationship between uncomplicated graft dilatation and structural failure has not been established. Consequently, there is no recommendation on a specific degree of dilation that constitutes a significant hazard and warrants graft replacement.

After implantation, a coagulum containing fibrin, platelets, and blood cells builds up during first few hours to days and stabilizes over a period of 6–18 months forming a compacted layer. The histological characteristics observed within Dacron grafts are a compact fibrin layer on the blood contacting surface and densely packed foreign body giant cells between the outer layer of graft wall and surrounding connective tissue capsule. The fibrin layer within the midgraft portion remains acellular regardless of whether the grafts are woven or knitted. Protein impregnation changes the surface properties of Dacron grafts and may induce more inflammatory reaction but does not change the clinical patency rates of these grafts.

Expanded polytetrafluoroethylene

PTFE was patented by DuPont in 1937 as Teflon, but its use as a vascular graft occurred in the 1960s as ePTFE. Bard and WL Gore have recently settled this patent case with each owning part of the ePTFE patent. Interestingly, this patent lawsuit has lasted from the 1970s into 2010s and may not be over yet [81].

The PTFE molecule is biostable and the graft made from it does not undergo biological deterioration within the body. The surface of the graft is electro-negative, which minimizes its reaction with blood components. ePTFE grafts are manufactured by stretching a melt-extruded solid polymer tube that then cracks into a non-textile porous tube. The characteristic structure of ePTFE is a node-fibril structure in which solid nodes connect through fine fibrils with average internodal distance (IND) of 30 μm for standard graft.

The initial host response to ePTFE grafts is similar to that of Dacron grafts [82]. A fibrin coagulum or amorphous platelet-rich material develops over a time sequence similar in both materials (Fig. 33.3). Interestingly, ePTFE may have some comparative resistance to certain infections in vascular procedures [83].

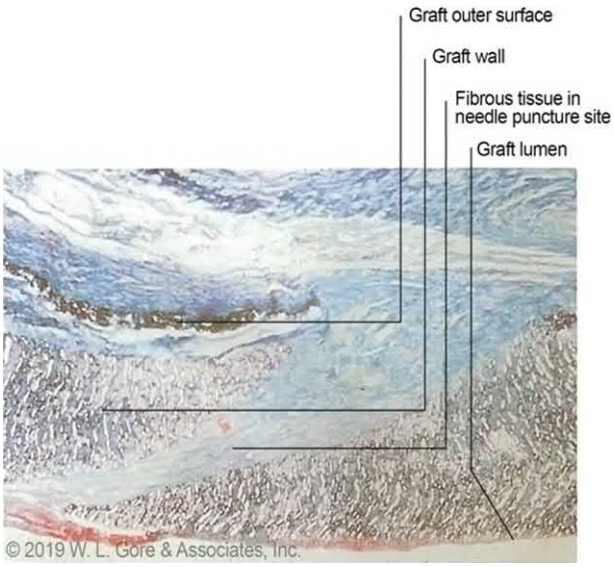

FIGURE 33.3 GORE-TEX integration. Representative image of host response to a needle entry site. Over time fibrous tissue incorporates into prosthetic grafts. *Image has not been previously published and is provided for use in this Chapter by W.L. Gore and Associates.*

Our improved understanding of these cellular and molecular components of biomaterial/tissue interactions has led many to pursue more intelligent designs of grafts that maximize beneficial ingrowth while minimizing the chronic inflammatory changes that lead to graft dilation or occlusion. These approaches include protein adsorptive grafts (growth factors, anticoagulants, antibiotics, etc.) as well as improved graft skeletal construction via synthetic polymers or biologically derived structural proteins that can be bonded to various bioactive cytokines and growth factors to induce a more favorable host response.

Vascular tissue engineering

Early efforts—in vitro tissue-engineered vascular grafts

Weinberg and Bell were the first to develop a TEVG in vitro [84]. Using collagen and cultured bovine vascular cells, they demonstrated the feasibility of creating a TEVG, but their graft had prohibitively low burst pressures, requiring external Dacron support. In the following decade, L'Heureux et al. constructed a human blood vessel with an acceptable burst strength and a thromboresistant endothelium in vitro using cultured umbilical cord-derived human cells [85], but because of the immunogenic effects of the heterogeneic ECs in vivo, this graft devoid of ECs had only a 50% patency rate at 8 weeks in a canine model. Since neonatal cells have a greater regenerative capacity, the above TEVG was not considered applicable to the aged population, who will derive the greatest benefit from a TEVG. In recent years, considerable progress has been made in the development and testing of TEVG.

Endothelial cell seeding

The prototype TEVG was created by seeding ECs onto ePTFE grafts in vitro, and then implanting them clinically. A confluent EC monolayer can prevent the development of myointimal hyperplasia by

1. preventing the deposition of platelets that release bioactive factors responsible for SMC migration, proliferation, and production of ECM;
2. maintaining a mechanical barrier to VSMC invasion via intimal basement membrane and the internal elastic lamina; and
3. assuming a quiescent EC phenotype that does not stimulate SMC activity.

In 1978 Herring et al. [86] first reported that EC seeding onto a graft surface enhanced graft survival in animal models. It was quickly discovered that the kinetics of EC loss following seeding showed that between 20% and 70% of initially adherent cells are lost during the first hour and as few as 5% were retained after 24 hours [87]. Retained cells at least partially compensate for the cell loss by migration and proliferation. Preconditioning the seeded EC monolayer with graded shear stress promotes reorganization of the EC cytoskeleton and production of ECM, which in turn enhances the EC retention at flow exposure [88]. In addition, Dacron and polyurethane have better cell attachment rates than ePTFE.

To maximize immediate cell inoculation density, a two-stage seeding procedure can be performed in which ECs are harvested, allowed one to proliferate in vitro to sufficient numbers, and then seeded and grown to confluence on the vascular graft prior to implantation. The disadvantages of this technique include the increased potential for contamination, the alterating of EC phenotype and function, the requirement of a 3–4 weeks waiting period for expansion of the cell population, and the necessity for two distinct procedures (first vein harvest for ECs; second the bypass). Zilla et al. demonstrated increased patency and decreased platelet deposition in clinically implanted EC-seeded (two-stage approach) ePTFE femoropopliteal bypass grafts over 3 years as compared to unseeded grafts, and this group more recently reported an overall 7-year primary patency rate of 62.8% for 153 endothelialized femoropopliteal ePTFE grafts [89]; this is comparable to the patency rate of saphenous vein grafts in this region. However, the seeded grafts have not been reproducibly shown to significantly reduce anastomotic pseudointimal hyperplasia. Despite the safety and efficacy demonstrated with this approach,

current use of this technology has been limited by stricter regulatory requirements.

There are also concerns about the ultimate function of those ECs on the graft surface, the cells having been injured by the process of manipulation and/or exposure to a nonphysiologic environment. Unlike their uninjured counterparts, injured ECs produce a variety of procoagulants such as von Willebrand Factor (vWF), plasminogen activator inhibitor, thrombospondin, and collagen. Higher levels of PDGF and bFGF have also been measured in EC-seeded grafts; this is particularly concerning, given their potential role in stimulating the migration and proliferation of SMCs, which can lead to IH.

In vitro approaches to tissue-engineered vascular grafts

The two main components of engineered arteries de novo are the cells and their scaffolds. There is much discussion about the proper cells to use for cell seeding ex vivo or cell homing in vivo. The EC is the most fastidious vascular cell to grow, and heterogenous ECs are highly immunogenic. Therefore in the absence of immunosuppression or genetic modification, autogenous ECs are thought to be a requirement for TEVG. TEVG media and adventitia can also be created by using vascular SMCs or fibroblasts with or without exogenous matrix scaffolding. These cells can be harvested from the patient in need, but since these patients are typically older with significant comorbidities, their cells (particularly VSMC and ECs) may not retain sufficient doubling capacity required to generate these TEVGs.

Niklason's approach of using healthy donor fibroblasts as the cell source obviates the challenges with autogenous but aged tissue that she has reported prior [90–92]. While quite a U-turn from prior work in cardiovascular tissue engineering, decellularized tissue scaffolds are appealing because they are already composed of native vascular ECM proteins that exhibit reasonable structural characteristics as well as providing instructive cues for cellular ingrowth. This is the current approach of Niklason's company, Humacyte, which is currently finishing phase 3 data collection with their TEVG as a hemodialysis conduit. It is an ECM derived from fibroblasts that are removed prior to processing the tissue (an acellular graft). These authors have recently published a certain degree of autogenous cell integration over time with progenitor cells in this construct when used for hemodialysis access [93] (Fig. 33.4).

L'Heureux et al. also created a suitable TEVG [94] that was shown to be a reasonable alternative for

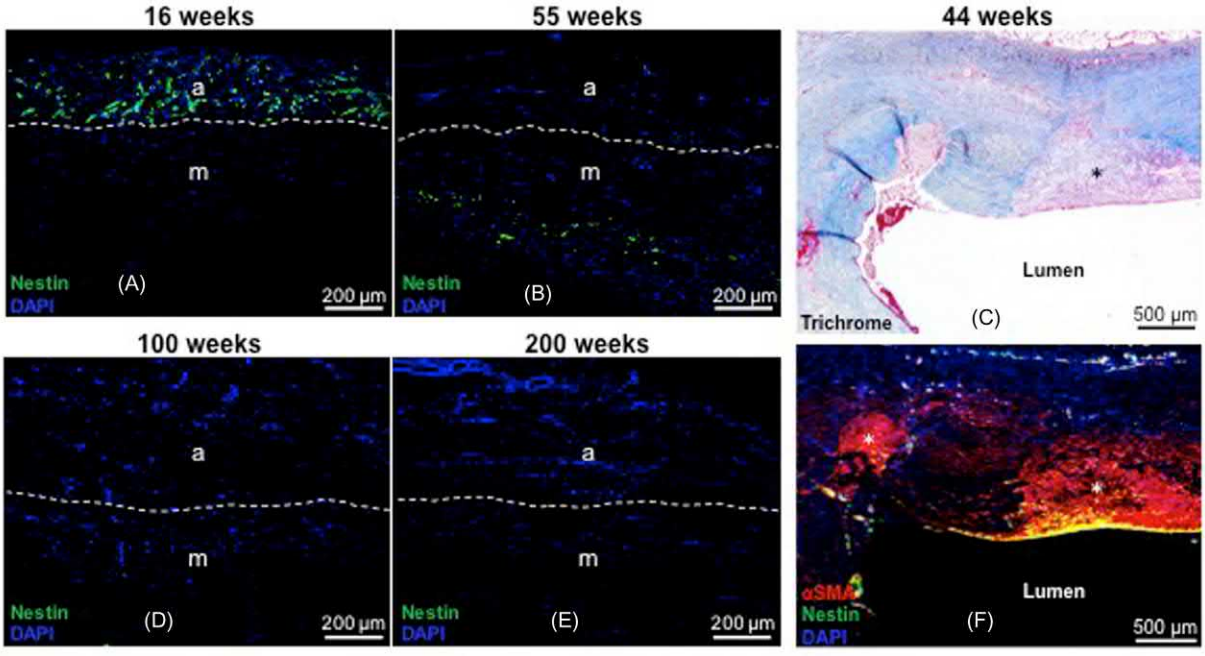

FIGURE 33.4 Autogenous cellular population of TEBV in dialysis patients. Representative immunohistochemistry and histologic stains of a TEBV from different patients harvest during reoperation at different time points. Nestin is represented in fluorescent green and DAPI in blue. (A and B) Nestin + progenitor cells are seen in two different TEBVs at 16 and 55 weeks after implantation. (C and F) Trichrome staining demonstrating "healing" of a needle puncture site. IHC staining demonstrating smooth muscle actin and Nestin + staining in the "healing" needle track. (D and E) Lack of Nestin + staining at 100 and 200 weeks after implantation. *TEBV*, Tissue engineered blood vessel; *IHC*, immunohistochemistry. *Image has not been previously published and was provided for this chapter by Laura Niklason's research group.*

hemodialysis use [95,96]. The L'Heureux laboratory has now demonstrated that critical components of the ECM are maintained during the devitalization process [97], which will likely be useful in developing and delivering novel devitalized cardiovascular tissue.

Using biologic gels, such as those composed of type I collagen or fibrin, one can promote tissue ingrowth and direct remodeling in a bioreactor, thereby promoting favorable characteristics such as improved mechanical strength or vessel reactivity over time [98]; these approaches are easily modified by the addition of growth factors with refined delivery systems in order to enhance and sustain cellular ingrowth [99]. Further refinement of these scaffolds can mimic the differential mechanical properties of the intimal and medial arterial layers. Syedian et al. reported development of TEVG from neonatal fibroblasts in a fibrin gel [100]. This promising approach has been updated recently with preclinical patency rates similar to that of some synthetic grafts [101], and the clinical testing of this approach is likely.

In vivo tissue-engineered vascular grafts

Current clinically available synthetic vascular grafts, composed of ePTFE, Dacron, or polyurethane are permanent prostheses within the host after implantation. Theoretically, it is possible with bioresorbable materials to stimulate a rapid and controlled ingrowth of tissue to assume the load bearing, sufficient for resistance to dilation, and to incorporate cellular and extracellular components with desirable physiologic characteristics to form a new artery in vivo, whereby the synthetic material itself would cease to be necessary following tissue ingrowth. Still, the limited regenerative capacity of aged or diseased cells discussed above may also compromise cellular ingrowth in vivo.

Bioresorbable grafts

Aneurysmal dilatation of bioresorbable grafts is the major limitation of this promising approach. This is due to the degradation of the graft material outpacing autogenous cellularization of the construct. The first published report of a fully bioresorbable graft was by Bowald et al. [102] and described the use of a rolled sheet of Vicryl (a copolymer of polyglycolide and polylactide). We have reported that 10% of woven polyglycolic acid (PGA) grafts have aneurysmal dilation within the first 3 months after implantation, but that this does not increase over the next 9 months, suggesting that the critical time for the development of aneurysms is during material resorption prior to the ingrowth of tissue. These studies also demonstrated the ability of bioresorbable grafts to support sufficient cellular ingrowth. Here, 4 weeks after implantation, these 24 mm by 4 mm grafts contained an inner capsule with a confluent layer of ECs and myofibroblasts amidst dense collagen fibers [103]. Similarly, constructed and implanted Dacron grafts demonstrated an inner capsule composed solely of fibrin coagulum with minimal cellularity. Macrophage infiltration and phagocytosis paralleled the resorption of PGA, which was totally resorbed at 3 months postimplantation.

In order to limit aneurysmal dilation, several approaches have been developed. One is to combine the bioresorbable material with a nonresorbable material in order to retain a mechanical strut. Another solution involves the combination of two or more bioresorbable materials with different resorption rates so that the more rapidly degraded material evokes a rapid tissue ingrowth while the second material provides temporary structural integrity to the graft. Third, growth factors, chemoattractants, and/or cells can be applied to the graft to enhance tissue ingrowth, structure, and organization.

Using a more slowly resorbed compound, polydioxanone (PDS), Greisler et al. demonstrated decreased incidence (1/28) grafts exhibited aneurysmal dilation with explant times as late as 1 year. The explanted specimens of these PDS grafts also demonstrated biomechanical characteristics similar to native arteries, being able to withstand static bursting pressures of 6000 and 2000 mmHg mean pulsatile pressure without fatigue [104].

Using the differentially resorbed approach with composite grafts woven from yarns of 74% PG910 and 26% PDS, we reported a 1-year patency rate of 100% with no aneurysms in a healthy rabbit aorta model. The PG910 was totally resorbed by 2 months and the PDS by 6 months. The regenerated arteries withstood 800 mmHg of pulsatile systolic pressure ex vivo without bursting, and a confluent, functional, vWF-positive EC layer over circumferentially oriented smooth muscle-like myofibroblasts formed in the inner capsule of both these grafts. Tissue ingrowth into all tested lactide/glycolide copolymeric grafts was observed to parallel the kinetics of macrophage phagocytosis and synthetic resorption. In vivo, the rate of cell proliferation and collagen deposition in inner capsule also paralleled the kinetics of macrophage-mediated synthetic resorption [105].

Composite grafts constructed from yarns containing 69% PG 910 and 31% polypropylene implanted into rabbit and dog arteries demonstrated superb results without aneurysmal dilation. Galletti used Vicryl (polyglactin 910) prostheses coated with retardant polyesters to temporarily protect the Vicryl from hydrolytic and cellular degradation [106]. When implanted into the canine aorta, material resorption was seen at 4 weeks and was complete by 24 weeks. In separate experiments, another group evaluated grafts prepared from a mixture of 95% polyurethane

and 5% polylactide [107]. They found that only relatively compliant grafts that induced circumferential smooth muscle development contained elastin and remained mechanically stable without dilating. They concluded that modifications of the graft preparation, including SMC seeding, help one to enhance the optimal orientation of the SMCs and prevent aneurysm formation.

There are a number of investigators currently pursuing this approach, but this approach seems further from clinical testing than decellularized constructs [108,109].

The living bioreactor

Campbell et al. have developed a modification of the Sparks' mandrel to create a TEVG. Here they utilize the abdominal peritoneum's reaction to foreign bodies as a living bioreactor [110]. After the graft has matured, it is removed from the mandrel and inverted; this creates a TEVG with a mesothelial inner lining. This graft's resistance to aneurysmal dilatation and rupture has not yet been proven, but Rotmans' laboratory is now using this in vivo approach to create tissue-engineered conduit for hemodialysis [111]. Clinical implantations have occurred and data should be forthcoming soon.

Cellular and molecular mediators of graft outcome

Cellular recruitment

Regardless of the approach, cellular recruitment occurs in all grafts (vein, synthetic, biologic), but perhaps it is most critical in decellularized constructs. Autogenous cells cannot populate an entire scaffold by migration or proliferation alone, they must be recruited internally via circulating ECs or EPCs or externally from the surrounding tissue or exogenous source through transmural angiogenic ingrowth (outside in radially) during tissue incorporation of the graft. In addition to the benefit provided by the localization of cells to the vessel lumen, transmural ingrowth also provides for a kind of "vasa vasorum" that can provide oxygen to cells beyond the distance supplied by simple diffusion (100 μm); such a *vaso vasorum* is desirable and could be incorporated into preexisting capillary networks (inosculation). Proof of concept of this approach has been demonstrated in cardiac sheet grafts [112]. The induction of a vaso vasorum can obviously be supplemented by the delivery of angiogenic proteins or genes to these constructs. With further research, a small diameter, totally resorbable vascular graft may be useful in creating a human artery replacement.

Physical or chemical modification of current grafts to improve durability

Since thrombogenicity and IH are the most common causes of graft failure and are both mediated at the luminal interface of the vessel or graft, the inner lining of grafts has been the subject of much investigation.

Surface characteristics

The thrombotic interaction at the grafts' luminal interface is dependent on both the chemical and physical properties of the graft (e.g., surface charge, surface energy, and roughness). A negative surface charge attenuates platelet adhesion and a positive charge promotes it, while a heterogeneous charge density distribution is also thought to be thrombogenic. A myriad of approaches have been designed to limit the thrombotic reaction, including modification of surface properties, incorporation of antiplatelet or anticoagulant substances onto the graft surface, and endothelialization of the luminal surface. In addition to thrombogenic reactions to the luminal surfaces, the rate and extent of endothelialization will vary depending on the characteristics of the surface [113]. Therefore properties must be optimized for both reduced thrombogenic reaction and maximized endothelialization.

Surface modifications

The simplest modification of a graft surface is to coat it with a relatively inert polymer. Since the 1960s, it has been known that carbon coating decreases surface thrombogenicity through its negative charge and hydrophobic nature. Experimentally, the carbon-impregnated synthetic graft was found experimentally to reduce platelet deposition, but the advantage of these grafts was not confirmed in a prospective multicenter clinical study of 81 carbon-impregnated ePTFE and 79 standard ePTFE grafts for below-knee popliteal and tibial/peroneal artery bypasses. Here the investigators failed to show a significant difference in patency rate between the two groups at up to 12 months after implantation [114].

Silicone polymer coating is another approach to alter the luminal surfaces of grafts; this process produces a smooth surface that is devoid of the usual ePTFE graft permeability and texture, and when followed by plasma glow discharge polymerization, it effectively abolishes pannus tissue ingrowth as well as graft surface neointimal hyperplasia in a baboon arterial interposition graft model. Further, Nojiri et al. [115] have developed a three-layered graft consisting of PET for the outer layer (to promote perigraft tissue incorporation), nonporous polyurethane in the middle layer (to obtain a smooth surface), and a 2-hydroxyethyl methacrylate and styrene copolymer coating for the inner layer (to establish a nonthrombogenic blood interface). These grafts with an ID of 3 mm were

implanted in canine carotid arteries and remained patent for over 1 year. Only a monolayer of adsorbed proteins was described on the luminal surface of the grafts with no pannus ingrowth from the adjacent artery, no thrombus, and no endothelial lining, or neointimal formation.

Thromboresistance

Early platelet deposition on vascular grafts is mediated by vWF and platelet membrane glycoproteins. After adherence to a graft, platelets degranulate, releasing many bioactive substances, including serotonin, epinephrine, ADP, and thromboxane A2. These substances in turn activate additional platelets and promote a prothrombogenic reaction. Activated platelets also release growth factors, such as PDGF, epidermal growth factor, transforming growth factor, which promote SMC migration and proliferation as well as ECM degradation and ECM protein synthesis. In addition, platelets release monocyte chemoattractants such as platelet factor 4 and β-thromboglobulin, which mediate the recruitment of macrophages to the graft. Platelet deposition and activation continues chronically after graft implantation as evidenced by increased thromboxane levels and decreased systemic platelet counts 1 year after Dacron graft implantation in a canine model [116], and human studies have confirmed platelet adhesion to grafts up to 1 year after implantation.

Since the deposition and activation of platelets elicits various pathologic cascades, the thrombogenic nature of the synthetic graft surface can lead to both early and late graft failure. A myriad of approaches have been studied to attenuate platelet deposition, aggregation, and degranulation. Antiplatelet agents directly targeting platelet/graft-binding molecules such as platelet surface GPIIb/IIIa and different functional domains of thrombin have been shown to at least transiently decrease the accumulation of platelets on Dacron grafts [117]. Also the surface thrombogenicity of grafts can be altered experimentally as described earlier.

Similar approaches can be utilized to decrease thrombogenicity by disrupting the activation of the blood system's coagulation cascade on thrombogenic surfaces, such as cardiovascular stents and synthetic grafts. These are now commercially available in various formulations. Genetic approaches to increase thromboresistance have been employed by multiple groups through the overexpression of thrombotic inhibitors, but since ECs themselves are antithrombotic, there may be limited benefit of this approach when compared to a functioning endothelium.

Heparin bonding to Dacron and ePTFE has shown promise in lower extremity bypass but has not been useful (over standard grafts) in hemodialysis [118]. Theoretical risks of heparin bonding, including the induction of heparin-induced thrombosis, have not been seen to date [119].

Protein adsorption

Another approach is to cover vascular grafts' lumens with proteins. Protein coating has been used as an alternative to preclotting with blood to decrease the initial porosity of Dacron grafts in order to limit transmural blood loss. Knitted Dacron prostheses coated with albumin, gelatin, and collagen have all been available for clinical use. As the impregnated proteins are degraded, the graft undergoes tissue ingrowth.

The most abundant serum proteins are albumin, fibrinogen, and IgG. They adsorb to grafts almost instantaneously following exposure to the systemic circulation. Subsequently there is a redistribution of proteins, known as the Vroman effect, according to each protein's relative biochemical and electrical affinity for the graft surface and their relative abundance [120]. Since platelets and blood cells interact predominantly with the bound proteins and not with the synthetic material itself, the constitution and concentration of bound protein has profound influence over the type and degree of cellular interaction with the graft. Fibrinogen, laminin, fibronectin, and vitronectin all have an arginine−glycine−aspartate (RGD) sequence that is recognized by platelets' glycoprotein (GPIIb/IIIa) receptor and initiate platelet activation. RGD sequences are also recognized by β_2 integrin, which directs leukocyte adhesion to the graft. Additional plasma proteins, including complement components, can also be differentially activated directly by different synthetic surfaces. For example, the generation of the monocyte chemoattractant, C5a, is greater following implantation of Dacron compared to ePTFE grafts in an animal model [121]. In addition, the rapid accumulation of coagulant proteins such as thrombin and factor Xa on the luminal surface after implantation contributes to the thrombogenicity of vascular grafts.

Porosity

The prevalence of open spaces or pores determines the porosity of a scaffold or synthetic graft, while the permeability of a graft is defined by its ability to permit passage of a substance through itself. Since ePTFE is composed of a number of solid nodes interconnected by a matrix of thin fibrils with no uninterrupted transmural spaces, it is best categorized by the average distance between these nodes, which is defined as the IND. This spacing, when above approximately 5−6 μm, as is commonly used in clinically available prostheses, allows for cellular ingrowth, but transinterstitial ingrowth is not strictly a function of porosity. We have shown that the extent of ingrowth varies greatly among different biomaterials

(e.g., PGA and Dacron) despite these biomaterials having similar porosity [122].

Still, the rate of tissue ingrowth can be improved by optimizing graft porosity or permeability. Clowes et al. have demonstrated enhanced tissue ingrowth and complete reendothelialization of 60 or 90 μm IND ePTFE grafts in a baboon model [123]. However, transinterstitial capillary ingrowth was not seen with the more commonly used 30 μm IND ePTFE. Human trials using ePTFE with these expanded INDs failed to show any advantage in platelet deposition compared to the standard 30 μm IND ePTFE grafts [124].

Compliance

The compliance mismatch between arteries and grafts causes flow disruption in vivo which may contribute to anastomotic pseudointimal hyperplasia [125]. It is for this reason that various surgeons have suggested interposing a segment of vein between the synthetic graft and artery, creating a composite graft at the distal anastomosis. This has led some investigators to design more compliant grafts using more flexible materials and/or changing the parameters of graft construction to improve graft compliance. Although animal experiments have suggested concept validity, the clinical benefit of this approach remains controversial. Many factors may contribute to this confusion, including longitudinal variability in the diameter and compliance of the arterial tree and the effect of activated endothelium on intimal hyperplasia. Further, there is a robust fibrotic response after implantation that leads compliant grafts to become incompliant after implantation; thus even if a compliance match were attained initially, it would not likely persist. In the *para*-anastomotic region there are dynamic changes in compliance that vary over time. First a *para*-anastomotic hypercompliant zone exhibits a 50% gain in compliance, then later its compliance is lessened 60% from baseline [126]. It is likely that this bimodal effect limits the practical value of this approach.

Resistance to infection

Vascular graft infection is rare, but it is catastrophic when it occurs; as demonstrated by an amputation rate of approximately 50% and a reported mortality rate that ranges from 25% to 75%. In an attempt to limit this dreaded complication, penicillin and cephalosporins have been successfully bound to Dacron and ePTFE grafts and found to limit *Staphylococcus aureus* infection in animal models. Rifampin-bonded gelatin-sealed Dacron grafts have also been shown in vitro to lessen bacterial colonization [127]. Intuitively, tissue ingrowth itself may also provide resistance to infection.

Graft infection is not only limb but also life threatening (Fig. 33.5). Interestingly, one of the benefits of the decellularized grafts being tested is resistance to infection. If this is demonstrated, it may drastically change clinical use of synthetics, even if patency rates are unchanged from synthetic [128].

Biological modification through exogenous sources

The delivery of potent angiogens or genes that promote EC-specific mitogenesis or chemotaxis upon synthetic surfaces may be used to regenerate a rapid and complete endothelium after vascular intervention. Such synthetics could store these genes or proteins and provide a controlled expression or release of these genes or proteins locally to circulating or surrounding ECs in a cell-demanded fashion. Ideally, this kind of synthetic would be available as an off-the-shelf alternative to autogenous vein.

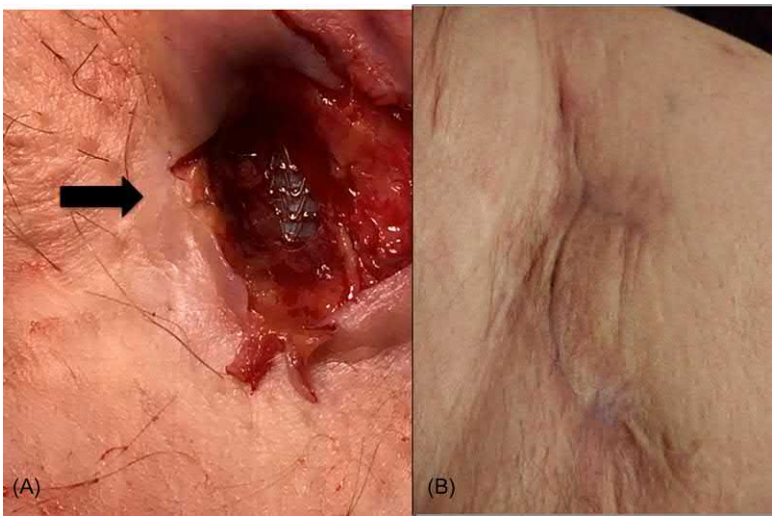

FIGURE 33.5 Exposed ePTFE stent graft. (A) Representative picture of a contaminated vascular graft after debridement in the operating room. (B) Here graft salvage was obtainable with a multidisciplinary medical and surgical team through aggressive debridement, focused antibiotic therapy, and a tissue flap. *ePTFE*, Expanded polytetrafluoroethylene.

Protein therapy

Although tissue incorporation is a desirable process for implanted prostheses, excessive vascular cell proliferation as well as ECM deposition can lead to intimal hyperplasia and ultimately graft failure. The ideal healing process in vascular grafts would be rapid endothelialization of blood contacting surfaces concomitant with a spatially and temporally limited subendothelial SMC growth, and followed by phenotypic and functional differentiation of cellular components and the subsequent remodeling of a mature ECM. The recent expansion of knowledge concerning the mechanisms responsible for the migration and proliferation of ECs and SMCs, angiogenesis, ECM deposition and remodeling, and physiologic parameters provides optimism for the possibility of manipulating the healing process through the directed manipulation of the microenvironment within the graft and perigraft tissue.

Since ECs have only limited capacity for regeneration, reendothelialization of the relatively large surface areas encountered clinically exceeds the normal mitogenic and chemotactic capacity of surrounding ECs. Thus endothelialization of large surfaces requires the recruitment of ECs from sites beyond the anastomotic border via the circulation or through transinterstitial migration from the surrounding tissue and/or the vasa vasorum. This is possible under the direction of localized angiogenic stimuli, and to a limited degree this is what occurs in vivo as protease-driven ECM changes and local availability of growth factors stimulate ECs, SMCs, and fibroblasts to enter the cell cycle.

The local delivery of growth factors or selected ECM may be utilized to promote desirable cellular events, such as endothelial ingrowth of synthetic grafts. For example, local delivery of exogenous angiogenic factors through a biologic delivery system (e.g., fibrin) may induce transmural capillary ingrowth in vivo, which can be the source of cells for an endothelial lining within synthetic grafts. The Greisler lab has evaluated the affixation of FGF-1 to synthetic surfaces. In early attempts, FGF-1 was applied to various synthetic grafts via a fibrin glue delivery system that, due to its structural orientation and state of polymerization, had been found not to be thrombogenic [129]. After delivering FGF-1 from fibrin glue to 60 μm IND ePTFE in both canine aortoiliac and thoracoabdominal aortic models, there was a significant increase in luminal EC proliferation as assayed by en face autoradiography, and a more rapid development of a confluent factor VIII positive endothelial blood contacting surface [130,131]. There was also extensive transinterstitial capillary ingrowth observed throughout the graft wall. Cross-sectional autoradiography did find a significant increase in subendothelial myofibroblast proliferation in these treatment grafts at 1 month, but this returned to baseline at later time points. Still, treated grafts developed a significantly thicker pseudointima (139 ± 178 vs 93 ± 89 and 67 ± 151 μm) at 140 days. In order to limit this IH response, we have developed site-directed mutants based on the FGF-1 angiogen and bioactive chimeric proteins that promote favorable characteristics, including prolonged bioactivity, EC specificity, or increased potency, while removing unfavorable characteristics such as heparin-dependent activity and susceptibility to thrombin-induced proteolysis [132,133].

Coimmobilization of FGF-2 and heparin in a microporous polyurethane graft by cross-linked gelatin has also been demonstrated to accelerate tissue regeneration on synthetic grafts, associated with a greater extent of endothelialization via perianastomotic and transmural capillaries ingrowth, in a rat aortic grafting model [134]. A consistent "neointima," approximately 40 m thick with intermittent endothelialization as well as SMCs and fibroblasts underneath the luminal surface were observed in the middle portion of treatment grafts, whereas the control grafts were only covered with a fibrin layer. However, because of the cross-talk that exists in the vessel wall between EC, SMC, and fibroblasts, multimodal therapies that promote EC coverage while limiting activation of VSMCs or the delayed delivery of cell cycle inhibitors may be required to optimize graft healing.

Gene therapy

Gene therapy shares the same promise as proteins, but it may also allow sustained or controlled protein expression in a desired location that is not possible with protein formulations. These qualities could obviate some of the current limitations encountered with the direct application of growth factor proteins to tissue beds for the regeneration of the endothelium.

This approach shows much promise as a delivery system, but single gene therapy trials have not yielded straightforward results. For example, although VEGF does improve endothelialization consistently, it does not reliably limit IH (and sometimes even promotes IH) in the literature. Gene therapy still requires cellular transduction or infection, and this has a variable effect on the cellular behavior. Also controversial results have been reported in the literature related to the proliferation, adhesion, and retention of genetically modified ECs on the surface of synthetic grafts. Another concern is that genetically modified ECs display poor retention on graft surfaces in vivo. This was demonstrated at 6 weeks in canine thoracoabdominal aortic ePTFE grafts seeded with lacZ-infected ECs compared to noninfected control ECs [135]. Further, Dunn reported only 6% retention of ECs that had been retrovirally infected with thromboplastin on Dacron grafts after 2 hours of exposure to flow in vivo [136].

For these reasons, little success has been documented to date concerning the long-term benefit of genetically modified EC-seeded grafts in vivo.

Cell therapy

Recruitment of EPCs into scaffolds provides a robust replication potential ideal for TEVG, and these cells acquire mature EC markers and function upon seeding into TEVGs [137]; these attributes may be further augmented by gene therapy.

In addition to the emphasis on the endothelialization of the flow surface, the function of other cell types, particularly the SMCs, in the vascular wall have become better appreciated. It has been suggested that ECs by themselves cannot produce a stable intima without SMCs or fibroblasts underneath. In support of this contention, tissue fragments containing multiple cell types, including venous tissue, adipose tissue, and bone marrow, have been seeded onto grafts and found to accelerate graft-healing processes.

Bone marrow–derived cells incubated on decellularized canine carotid arteries by Kim et al. demonstrated cellular incorporation into the scaffold and subsequent differentiation of these cells into endothelial and vascular SMCs and subsequently into three distinct vessel layers [138]. Similarly adipose-derived stem cells have been used successfully as a cell source for TEVG by a number of investigators [139,140]. However, at least in adipose-derived stem cells, it appears that diabetic donors severely compromise TEVG patency [141]. More recently, Brewster et al. have demonstrated robust secretome profiles from both bone marrow and adipose tissue of diabetic patients with PAD and PAD alone [142,143], suggesting that MSCs (at least after culture) retain many of their desirable paracrine properties.

Interestingly, bone marrow cell seeding was also reported to induce an abundant capillary ingrowth in the graft wall and a rapid, complete endothelialization of the inner surface without intimal hyperplasia. Since the bone marrow stem cells have the ability to differentiate in response to their microenvironment and to proliferate as well as secrete cytokines critical to their survival, they may provide a useful cell source for blood vessel tissue engineering.

Conclusion and predictions for the future

Much progress has been seen in recent years, and yet old things also become new again. Since the publishing of the last chapter, the decellularized "nude" implantation of a decellularized TEVG appears to be what will be clinically available first. Phase III publications will be coming forth soon to confirm this. However, promising tissue engineering approaches using bioresorbable grafts and/or the intelligent induction of a vaso vasorum into TEVG or existing synthetic grafts will likely be version 2.0 for TEVG work. Likely, these next generation TEVGs will be tested in clinically relevant animal models, as their path to clinic will likely require this.

Finally, as more patients live with cardiovascular disease than die from it, there is little doubt that promotion of durable graft healing will be more effective than blockade strategies of pathologic processes such as IH. However, both strategies, as well as combinatorial strategies, will likely yield new insights and problems for future investigators. Such a rich clinical need worldwide creates a sustained need for research investment. Those who persist and involve clinical trialists in the development of their TEVG will have the greatest chance of bringing reality to the dream of blood vessel replacement [144].

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Chapter 34

Heart valve tissue engineering

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Introduction

Heart valve function and structure

The physiological function of a heart valve is to maintain unidirectional nonobstructed blood flow without damaging blood elements, causing thromboembolism, or placing excessive mechanical stress on the leaflets and cusps. The native heart valve is remarkably well adapted to performing these functions. This capability arises from a near perfect correlation of structure to function, enabling the valve to avoid excess stress on the cusps while simultaneously withstanding the wear and tear of 40 million repetitive deformations per year, equivalent to some 3 billion over a 75 year lifetime [1]. There are four valves in the human heart: two semilunar and two atrioventricular (AV). The semilunar heart valves represent the outflow tracts of the heart and include the structurally similar aortic and pulmonary valves. The AV heart valves include the tricuspid and mitral valves, which separate the atrium from the ventricle on the right and left side of the heart respectively. The following text describes the relation of structure to function in the semilunar valves from a macroscopic (tissue) to microscopic (cellular) level.

Grossly, the semilunar heart valves are composed of three thin cusps that open easily when exposed to the forward blood flow of ventricular systole, and then rapidly close under the minimal reverse flow of diastole [1]. The three cusps of the aortic valve are the left, right and noncoronary cusps. Each cusp is attached to the aortic wall by a thick base known as a commissure. Despite the force applied to the leaflets during diastole, prolapse is prevented by substantial coaptation of the cusps in a crescent shaped region of the cusp termed the lunula. In addition, the structural elements within the aortic valve cusps are anisotropically oriented in the tissue plane, resulting in disproportionate mechanical properties of the valve cusps with greater compliance in the radial rather than the circumferential direction. This compliance allows the cusp thickness of the aortic valve to vary from 300 to 700 μm throughout the course of the cardiac cycle [2]. Further structural specializations that occur are lengthwise folding of collagen fibers, and orientation of collagen bundles in the fibrous layer toward the commissures. This orientation conserves maximal coaptation, and thereby prevents regurgitation. Thus, both the macroscopic valve geometry and the fibrous network within the cusps work to transfer stresses caused by the diastolic force to the aortic wall and annulus.

Microscopically, the semilunar heart valve is composed of three layers: the ventricularis, spongiosa, and fibrosa (Fig. 34.1). It is the unique extracellular structural characteristics within these layers that create a specialized biomechanical profile necessary for proper function. The fibrosa, which is exposed to the aortic lumen, is composed of primarily collagen fibers, which are densely packed and arranged parallel to the cuspal free edge. It is these collagen fibers, mostly types I and III, which provide most of the mechanical strength of the valve [3]. The ventricularis layer faces the ventricle and is composed of collagen and radially aligned elastin fibers. Elastin forms an encompassing matrix that binds the collagen fibrous bundles throughout the heart valve, thereby creating an elastin-collagen hybrid network of interconnected fibers that provides greater mechanical strength [4]. The centrally located spongiosa layer is composed of glycosaminoglycans (GAG) and loose collagen fibers. The GAG side-chains of proteoglycans make a gelatinous substance in which other matrix molecules are able to form covalent crosslinks that support other components of the extracellular matrix (ECM) [5]. Human heart valve GAGs are

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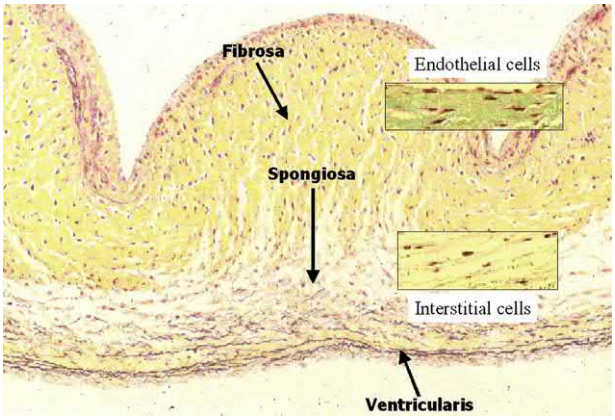

FIGURE 34.1 Histological cross section of a heart valve demonstrating the ventricularis, spongiosa and fibrosa. Also shown are interstitial cells and endothelial cells.

composed predominantly of hyaluronic acid, with smaller amounts of dermatan sulfate, chondroitin-4-sulfate and chondroitin-6-sulfate [6].

Cellular biology of the heart valve

Valvular endothelial cells

The two most prevalent cell types in heart valves are valvular endothelial cells (VECs) and valvular interstitial cells (VICs). VECs line the surface of valve cusps on both the aortic and ventricular surfaces. They provide a nonthrombogenic blood-tissue interface and maintain a semipermeable membrane that regulates the transfer of large and small molecules through the vascular wall [2]. Endothelial cells are also critical in the control of inflammatory and immune reactions, in regulating the proliferation of other cell types, and play a role in metabolism and protein synthesis [7].

The role of VECs in the heart valve is not passive. VECs can respond to external stimuli by phenotypically changing and acquiring new, inducible properties through a process known as endothelial activation. Both cytokines and hemodynamic forces have been shown to induce this process [2]. Once activated, VECs produce cytokines, chemokines, adhesion molecules, growth factors, and vasoactive molecules, all of which contribute to valve adaptation in response to the initial stimuli. Most often, this process is beneficial and allows for maintenance of valve homeostasis. However, a form of endothelial activation can occur that is known as endothelial dysfunction, which results in a surface that is adhesive to inflammatory cells or thrombogenic and can be a source of valve pathology [2].

Although VECs are often grouped together, there is in fact heterogeneity among these cells, as there is among endothelial cells in general. Their source, be it arterial or venous tissue, can affect cell phenotype, as can their location (aortic or pulmonary) [8]. Moreover, there is an increasing appreciation for the substantial data suggesting that VECs differ in many ways from other types of endothelial cells. For example, in response to mechanical stress, VECs align perpendicularly to blood flow, whereas vascular endothelial cells in the aorta align in parallel with flow [9]. In a separate study, investigators demonstrated significantly different transcriptional gene profiles of VECs on aortic and ventricular sides of porcine aortic valves [10].

Valvular interstitial cells

VICs are the most abundant cell type in heart valves and are primarily responsible for heart valve stability by the synthesis and remodeling of valve ECM. This process maintains the appropriate structural and functional relationship that allow for long-term heart valve competence. VICs contain varying characteristics of myofibroblasts, fibroblasts, and smooth muscle cells. There are five subtypes of VICs: embryonic, progenitor, quiescent, activated, and osteoblastic. Each subtype is unique and VICs can change phenotypically from one to another depending on the mechanical environment, chemical cellular or hormonal signals, or in response to injury. Embryonic VICs undergo endothelial-to-mesenchymal transformation (EMT), initiating the formation of the valve in the embryo [11]. Progenitor VICs represent a heterogeneous cell population that is thought to be important to valve repair [11]. Osteoblastic VICs regulate the process of calcification within the valve [11].

The two most important VIC populations to valve function are those that are quiescent and those that are activated [5]. In the quiescent state, VICs most closely resemble fibroblasts in that they have low levels of α-smooth muscle actin [12]. VICs can be activated by a variety of different stimuli and conditions, including during embryonic valve development and with exposure to TGF-β or cyclic stretch. In addition, various valvular pathologies can induce VIC activation. Importantly, studies have shown a distinct transition from quiescent to active VICs after implantation of a tissue engineered heart valve (TEHV). Similar results were seen after implantation of a pulmonary autograft as an aortic valve replacement [13].

VICs are of central importance to heart valve repair and maintenance operations. The constant and rigorous mechanical movement of valves results in persistent low level valvular damage and an ongoing ECM synthesis, remodeling, and degradation process. VICs regulate and perform this process by expressing ECM components and the proteins involved in matrix remodeling like matrix metalloproteinases (MMPs) and tissue inhibitors of

metalloproteinases (TIMPs). Importantly, over-expression of MMPs has been observed in several heart valve pathologies, indicating a possible target for clinical diagnostic and predictive markers as well as for therapeutic intervention [14,15]. More investigation is needed to better understand the mechanism of action of MMPs and TIMPs and their importance in valve tissue morphogenesis, repair and remodeling.

Even though ample data exists regarding the reparative properties of VICs, their function is still not fully understood [5]. Important to understanding and potentially manipulating VIC phenotype and cellular activity is knowing how these cells send and receive signals, how mechanical information is transmitted from the ECM, and what specific factors can induce a variety of cellular responses like cell migration, adhesion, growth, and differentiation [16].

Innervation and vasculature

While the VICs and VECs make up the majority of the cellularity of the valves, nerves and vascular networks are also present and worthy of attention. A large number of nerve cells have been demonstrated in valve leaflets, particularly in the aortic valve, and they have been shown to impart some control over the mechanical properties of the valve [17]. The nerves are present primarily on the ventricularis side of the valve, and are present throughout nearly the entire valve surface, except for being noticeably absent on the coapting edges of the leaflets [18]. The pulmonary valve is innervated similarly to the aortic valve, while the AV valves are more heavily innervated, being primarily innervated on the atrial side with innervation including the entire surface up to the coapting edges as well as through the chordae tendineae [19,20]. This nervous system control may allow for the mechanical properties of the valve leaflet to change throughout the cardiac cycle to better adapt to the differing conditions present throughout the cardiac cycle.

The vasculature also differs between the AV and semilunar valves. Aortic and pulmonary valve leaflets are thin enough to be perfused from the surrounding blood and as such contain few blood vessels. Mitral and tricuspid leaflets, on the other hand, contain capillaries in their most basal portion to aid in perfusion, with some small vessels also present in portions of the chordae tendineae [21].

Heart valve dysfunction and valvular repair and remodeling

Heart valve dysfunction

The American Heart Association has estimated that over 300,000 heart valve replacement procedures were performed in the United States between 1998 and 2005 [22]. Population growth, population aging, and improvements in longevity will fuel demand for heart valve replacements in the future, with a projected need of 850,000 in year 2050 [23]. The pathology of heart valve disease manifests itself in two ways, the first being a reduction in forward flow caused by failure of a valve to completely open, known as stenosis. The second is back flow during diastole caused by failure of a valve to completely close, known as insufficiency or regurgitation.

Aortic stenosis is a common valvular pathology. When severe, this disease can cause dyspnea on exertion, chest pain, and syncope. The most frequent cause of aortic stenosis is dystrophic calcification of the aortic valve cusps and ring (annulus). Overall prevalence of aortic stenosis in the United States is 2% and is increasing with time as the American population ages. Bicuspid aortic valves are the leading cause of aortic stenosis in younger patients, and represent one of the most frequent congenital cardiovascular malformations in humans, affecting approximately 0.5% of the young-adult population [24]. Bicuspid aortic valves are also sometimes associated with aortic insufficiency and increase the risk of infective endocarditis. Through retrospective community studies of patients with an identified bicuspid aortic valve, the total combined risk related to the bicuspid aortic valve, including aortic regurgitation, surgery, and infective endocarditis, was 52% for men and 35% for women [24]. The main source of chronic aortic insufficiency is aortic root dilation. This results in distended and outwardly bowed commissures as well as impaired cuspal coaptation.

Mitral valve prolapse is defined as the displacement of valve leaflets into the left atrium during systole and is the most common indication for surgical repair or replacement of this valve. Mitral valve prolapse is most commonly caused by myxomatous degeneration, identified by a diffuse build-up of GAGs within the valve, and is also seen in patients with Marfan syndrome. Patients with mitral valve prolapse are exposed to increased risk of a variety of different complications including heart failure, bacterial endocarditis, mitral regurgitation, atrial fibrillation, and thromboembolic events [7]. The most common cause of mitral stenosis worldwide is rheumatic fever, which can cause lasting damage to the mitral valve leaflets, leading to leaflets thickening and fusing later in life [2].

The leading cause of dysfunction of the tricuspid and pulmonary valves is congenital heart disease. Approximately 20,000 infants are born in the United States each year with a congenital heart defect, of which many involve absence or malformation of the pulmonary valve and pulmonary artery [25]. Major congenital heart disease diagnoses of the right ventricular outflow tract (which includes the pulmonary valve and pulmonary artery) include truncus arteriosus, pulmonary atresia with

ventricular septal defect, severe Tetralogy of Fallot, transposition with ventricular septal defect and pulmonary atresia, and double-outlet right ventricle. It is these children who would benefit most from TEHVs, which offer the potential to grow with the child thereby preventing need for re-operation.

Valvular repair and remodeling

When valvular injury occurs, it stimulates VIC proliferation, migration, and apoptosis. The initial events in valve repair of a linear superficial denuding wound have been modeled in vitro and are characterized by prominent migration and proliferation of VICs [26]. The migration of VICs is governed by a sequence of processes including activation of integrins, which are cell surface heterodimeric receptors that control cell−ECM and cell−cell adhesion [27]. Changes in macroscopic mechanical stimuli like shear and solid stresses are also propagated via signal transduction through a complex cell−ECM network, which allows small external changes in valvular mechanics to ultimately affect cellular activity and function [7].

Activation of VICs results in a change of phenotypic subtype to activated myofibroblasts, as demonstrated in leaflets from patients with myxomatous mitral valve degeneration [14]. These phenotypic changes resemble the evolution of physiological wound healing in mitral valves associated with the phenotypic modulation of interstitial valvular cells from fibroblasts to myofibroblasts [2]. That said, when equilibrium is reached after injury, cells revert back to a quiescent phenotype like that of a fibroblast. However, in cases in which there is persistent injury or where a steady state equilibrium cannot be achieved, VICs will remain persistently activated, contributing to ECM remodeling and initiation of valvular pathologies [28].

Important to understanding VIC function and activation in response to injury is the interaction between VICs and the ECM. VICs takes cues from and act upon ECM by secreting ECM components and their proteolytic enzymes, and by promoting avenues of cellular migration. Interstitial collagenases, gelatinases and other MMPs are involved in the degradation and remodeling of connective tissue and are primarily secreted by activated VICs [29]. As in tissue throughout the body, MMPs in heart valves are critical in tissue morphogenesis, wound healing, and other tissue remodeling processes. The interaction between MMPs, their inhibitors (TIMPs), and their regulators are particularly important in cardiac and vascular remodeling [2]. In the degradation of ECM, interstitial collagenases MMP-1 and MMP-13 mediate the preliminary phase of collagen breakdown by disassembling the native helix of the fibrillar collagen. The resulting collagen fragments are then accessible to further proteases, like gelatinases [30]. These interstitial collagenases are secreted by activated VICs in a similar manner as they are secreted by inflammatory cells in a multitude of systemic diseases [2]. That is, VICs are being stimulated by signal transduction, be it mechanical, chemical, hormonal, or otherwise, to produce and secrete soluble ECM proteases that allows for remodeling of ECM in response to changes in external environment. One means of signal transduction is cardiac catabolic factor, which is derived from porcine heart valves and found to stimulate collagen and proteoglycan breakdown in vitro [31]. While MMPs are a necessary component to normal remodeling of heart valves, excessive levels of MMP activity can lead to excess collagen and elastin breakdown, thereby weakening heart valve leaflets and predisposing to disease [14].

Heart valve replacement

Standard treatment for end-stage valvular dysfunction is heart valve replacement. The first successful implantation of a human valve was performed in 1952 [32]. Since then, more than 80 different designs of prosthetic heart valves have been developed [33]. Heart valve substitutes have undergone a progressive evolution as newer models are developed to remedy the deficiencies of older devices. Prosthetic heart valves are either mechanical and composed entirely of synthetic material, or bioprosthetic and therefore fashioned from biological components. Slightly more than half of the world's implanted valves are mechanical, while the remainder are bioprosthetic [34]. While each type of heart valve is used successfully to improve the quality and length of life, each valve type also has its own unique set of problems [35]. The overall rate of complications is similar for mechanical prostheses and bioprostheses [36]. Four categories of valve-related complications predominate [1]:

1. Thromboembolism, thrombosis, and secondary anticoagulation-related hemorrhage;
2. Prosthetic valve endocarditis;
3. Structural dysfunction including failure or degeneration of the prosthetic biomaterials; and
4. Nonstructural dysfunction including complications arising from technical problems during surgical implantation such as perivalvular leak and biological integration (tissue overgrowth).

Each valve type is associated with its own unique set of advantages and disadvantages. The mechanical heart valve is characterized as having excellent durability due to the mechanical properties of the synthetic materials from which it is constructed. The material properties of mechanical heart valves allow them to last longer than 15 years following implantation [37]. Unfortunately, these synthetic materials also give rise to poor biocompatibility.

Specifically, mechanical prosthetic valves are associated with a substantial risk of thromboembolism and thrombotic occlusion caused by the lack of an endothelial lining and the flow abnormalities that result from a rigid outflow structure [38]. To minimize this risk, chronic anticoagulation therapy is required for all mechanical valve recipients. However, systemic anticoagulation renders patients vulnerable to potentially serious hemorrhagic complications. Thus, the combined risk of thromboembolic complications and hemorrhage secondary to anticoagulation constitute the principal disadvantage of mechanical prosthetic valves. A metaanalysis found an incidence of major embolism in the absence of antithrombotic therapy of 4 per 100 patient years, which was decreased to 2.2 per 100 patient years with antiplatelet therapy and to 1 per 100 patient years with anticoagulant therapy (e.g., warfarin) [39]. However, long-term anticoagulation is not without its own risks. Disadvantages of long-term warfarin use include increased risk of bleeding complications, lifetime need for blood tests and therapy to maintain a therapeutic international normalized ratio, and high rates of noncompliance [40]. In a review of randomized trials of anticoagulation therapy in patients 65 years and older, incidence of major bleeding ranged from 0% to 4.6% per year, with incidence of minor bleeding as high as 10.5% per year [41]. Indeed, patients over the age of 75 on anticoagulation for mechanical heart valves compared to patients who received a bioprosthetic valve assessed for bleeding rates had an odds ratio of 18.9 [42]. Mechanical heart valves are also prone to fibrotic ingrowth and regurgitation [43]. As mechanical heart valves do not undergo biodegradation or remodeling, any tissue growth onto the valve components over the life of the implant can contribute to compromising the valve performance [44]. Other disadvantages of mechanical heart valves include their ability to cause hemolysis and an increased susceptibility to endocarditis [45].

Bioprosthetic valve replacements such as glutaraldehyde-fixed xenografts and allografts are associated with a lower risk of thrombosis and hemolysis than mechanical heart valves [35]. Patients with glutaraldehyde-fixed xenograft valves do not require anticoagulation and therefore do not incur the risks of anticoagulation-associated bleeding. However, because of their mechanical properties and their composition of biologic material, the durability of a glutaraldehyde-fixed valve is more limited than that of the mechanical valve. The major disadvantage of tissue valves is progressive structural deterioration that eventually results in stenosis and/or regurgitation. The degradation mechanisms of bioprosthetic valves are progressive and the rate of failure is highly time dependent.

Alterations of their molecular composition and/or tissue structure of bioprosthetic valves during manufacturing can lead to valve failure [1]. One such example is the permanent fixture of one or more valve cusps in a specific configuration during manufacture which is associated with only one specific phase of the cardiac cycle [2]. In this case, normal cyclic rearrangements in the valve subarchitecture cannot appropriately occur and irregular tissue stress is created. Other examples include process-induced destruction to the endothelial coating which then allows penetration of inflammatory cells and plasma into the cusp after implantation, leading to valve inflammation-induced thickening and possible valve deterioration [2].

The principal problems for bioprosthetic valve durability after implantation are cuspal mineralization and noncalcific mechanical fatigue. Calcification occurs when calcium from plasma binds with residual organic phosphates of the crosslinked, nonviable cells of the preserved valve [7]. Further weakening can occur from proteolytic degradation of the collagenous ECM [46]. MMP activity has been demonstrated in explanted valve tissue that has undergone structural degradation, which signifies a degree of inflammation and ECM remodeling within damaged valves [47]. Similar findings have been found in in vitro models of valve function, suggesting that the mechanical stress that bioprosthetic valves are subject to may alone contribute to ECM degradation and prolonged remodeling [48].

Despite improvements over the last several years in bioprosthetic valve durability, structural valve deterioration is a significant problem particularly in patients less than 65 years old. In one study looking at patients having had a bioprosthetic aortic valve replacement, 60% of patients younger than 65 years required re-operation by 18 years postimplantation [49]. Indeed, the risk of structural failure is strongly age dependent, with individuals less than 35 years of age, and especially children and adolescents, having the highest rate. Nearly uniform failure occurs by 5 years in those less than 35 years old but 8%–27% fail in 20 years in those older than 65 [50]. The major cause of bioprosthetic valve dysfunction is structural deterioration of the cuspal tissue [51]. Two distinct yet potentially synergistic processes are causal: calcific degradation [1] and noncalcific degradation [2]. Both eventually lead to failure of the connective tissue matrix of the tissue valve [1].

Another type of bioprosthetic valve is the cryopreserved homograft, which is particularly advantageous in those patients requiring aortic valve replacement or those needing congenital heart reconstruction with right-sided conduits [52]. However, the use of the cryopreserved homograft is a form of transplantation and thus subject to many transplant-associated problems, including the implanted tissue invoking a host immune response, thereby potentially increasing the risk and rapidity of

structural valve deterioration. Cryopreserved homografts are currently the most biocompatible replacement heart valve and the treatment of choice in most pediatric cardiothoracic applications [53]. However, used in this application, they are severely limited by their inability to grow and by a significant incidence of structural deterioration resulting in limited durability and frequent need for reoperation. Overall, the poor long-term durability of currently available heart valves in young patients (children and adults <35 years) makes clear the clinical need for an improved replacement heart valve, a need which tissue engineering can help fulfill.

A recent advancement in the field of heart valve replacement is the transcatheter approach to heart valve deployment, first performed in a human patient in 2002, with over 250,000 procedures being done to date [54,55]. This technique consists of a valve within a stent that is delivered through a catheter, and may be performed as an initial valve replacement therapy or as a follow-up therapy to replace a failing bioprosthetic valve, referred to as a valve-in-valve procedure. Transcatheter deployment carries the benefit of being minimally invasive, particularly compared to a traditional open surgical approach, allowing for the option of valve replacement in high risk patients who may not be eligible for open surgery. Transcatheter valve replacement is not without its own risks, however. Transcatheter valve deployments have shown higher rates of perivalvular leak, cerebral embolism, and conduction anomalies requiring pacemaker implantation than surgical valve implantation [56].

The application of tissue engineering toward the construction of a replacement heart valve

Tissue engineering theory

The ideal heart valve replacement would be perfectly biocompatible, readily available, durable, and have the potential for growth. The construction of an autologous, TEHV could potentially fulfill all of these requirements by utilizing natural mechanisms for repair, remodeling, and regeneration. The central paradigm underlying tissue engineering involves combining cells with a platform matrix to create neotissue [57]. The matrix acts as a three dimensional scaffold until proliferating cells produce sufficient ECM in vitro to permit in vivo implantation [2]. This process is followed by scaffold degradation, neotissue formation, and growth.

Tissue engineering is currently limited by the inability to construct microvasculature de novo, the inability to control innervation of neotissue, and the difficulties surrounding culturing certain cell types [58]. Although the semilunar heart valve is not totally avascular, oxygen and nutrients needed to sustain its function are supplied via two complementary pathways: diffusion from the blood stream and via a capillary network. In addition, semilunar heart valves are composed of cells that are readily grown in culture. This allows for the isolation and expansion of autologous cells for construction of TEHVs. The blueprint for constructing an ideal TEHV has evolved from a large body of research in bioprosthetic valves, diseased heart valves, and other tissue valve substitutes. Although these investigations have largely been clinical in nature, they have identified useful markers of cell function, matrix physiology, and matrix structure. These results led to several key interrelated concepts of functionally adaptive valvular remodeling and regeneration [2]:

1. Valves are composed of a highly specialized arrangement of collagen and other ECM components;
2. Native and substitute valves are subject to structural deterioration over time;
3. The generation of new valvular ECM is dependent on VIC viability and function.

Therefore, the long-term success of a tissue engineered valve replacement is theorized to depend on the ability of its living cellular components, whether seeded cells or infiltrating host cells, to assume normal function with the capacity to repair structural injury, remodel the ECM, and potentially grow.

Tissue engineering offers the potential to create a nonthrombogenic, biomimetic, immunologically compatible tissue valve substitute that is capable of providing ongoing remodeling and repair which would allow growth in maturing recipients (Fig. 34.2) [2]. Such a technology would have dramatic implications in a clinical setting and greatly contribute to improving patient outcomes.

Biomaterials and scaffolds

Over the last several decades, an exhaustive variety of biomaterials have been explored to serve as scaffolds for TEHVs. These biologic scaffolds have the primary responsibility of promoting tissue regeneration and must be biocompatible, biodegradable into safe byproducts, easily manufactured and handled, highly porous to facilitate cell attachment and infiltration, and yet mechanically stable enough to appropriate function [2].

Scaffolds can be manufactured from either synthetic or natural materials. Natural biomaterials include ECM components such as collagen, fibrin, elastin, GAG, or decellularized tissues, such as heart valve, pericardium, arterial wall or small intestinal submucosa [60]. Synthetic polymers have an advantage in that they have predictable chemistry and their properties can be well-controlled [2]. In addition, these materials are FDA (Food and Drug Administration) regulated and many have been

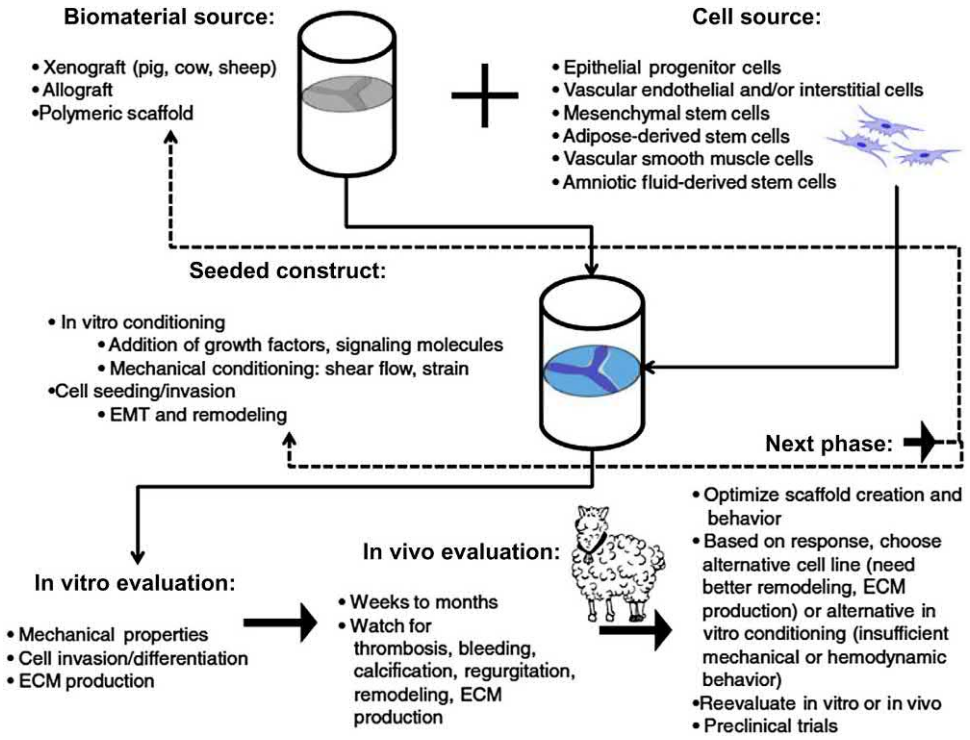

FIGURE 34.2 Overview of tissue engineering heart valves [59].

approved in clinical applications [59]. The most commonly utilized synthetic polymers in tissue engineering include poly(glycolic acid) (PGA), poly(L-lactic acid), copolymer poly(lactic-*co*-glycolic acid) (PLGA), poly (ethylene glycol), and polyhydroxyalkanoate.

A number of natural biodegradable polymers have been explored for use as scaffolds. They have the advantage of offering a more biocompatible template on which cells grow [5]. Decellularized small intestinal submucosal (SIS) matrix has been isolated to use in tissue engineering [61]. Other investigators have used porcine SIS scaffold as a resorbable matrix to make pulmonary valve leaflet replacement in porcine models [62]. When the explanted constructs were evaluated they revealed resorption of the submucosal matrix, fibrous connective tissue growth and formation of neo-vasculature.

Much of a heart valve's mechanical and tensile strength originates from its collagen composition and alignment [63]. Due to their highly conserved genetic code across species, collagens are weakly immunogenic and have been used widely for TEHV constructs seeded with both human and porcine-derived cells [64,65]. Another natural scaffold that has been explored is fibrin gel. It has been used as an autogeneic scaffold in heart valve tissue engineering [66]. Some advantages of using fibrin gel as a material for scaffold construction include that it is made from the patient's own blood, and also that it is readily biodegradable. However, early attempts at utilizing fibrin gel were limited by its tendency to shrink and its inability to withstand surgical implantation [5].

The process of removing cells from a biological matrix can reduce the immune response to the bioprosthetic while maintaining the potential for endothelialization by host VECs and VICs, either through cell ingrowth or via cell seeding [67]. Methods of tissue decellularization range from enzymatic digestion, detergent treatment, sonication and hypo-/hypertonic immersion [68]. These treatments effectively remove antigenic components such as nucleic acids, cell membranes, cytoplasmic structures, lipids, and soluble matrix molecules but retain elastin, collagen and GAG components of the scaffold ECM [69]. Decellularization has been utilized in porcine matrices that were subsequently seeded with human endothelial cells. The resulting construct had a confluent and viable monolayer cell surface, an important feature in reducing thrombogenic risk [70]. Further experimentation in vivo utilized a decellularized pulmonary valve seeded with endothelial cells and carotid artery myofibroblasts followed by implantation in an ovine model [71]. After 1 month, the valve leaflets were completely endothelialized with dense infiltration by myofibroblasts by 3 months. However, enthusiasm was tempered by the finding of subvalvular calcification and inflammation resulting in increased thickening of the valve leaflets [5].

The commercially available SynerGraft valve (CryoLife Inc., United States) is based on the decellularization model of tissue engineering [72]. The SynerGraft decellularization process involves cell lysis in sterile water, enzymatic digestion of nucleic acids and a multiday isotonic washout period. The method was developed as an alternative to glutaraldehyde crosslinking to decrease xenograft antigenicity. Histological evaluation 150 days postimplantation in porcine models showed intact leaflets with local myofibroblasts ingrowth and no calcification [5]. Early failure of the valve, however, was reported in human trials [73]. The SynerGraft matrix initiated an early strong inflammatory response followed by a lymphocyte response. Rapid structural degeneration of the valves occurred within 12 months. Furthermore, the valve scaffold was not repopulated with host cells, and calcific deposits were detected.

A comparison of decellularized cryopreserved allografts to traditional cryopreserved allografts by Burch et al. showed while there were no differences in degree of insufficiency, the decellularized allografts had a minor improvement in pressure gradient over their traditional counterpart [74]. However, Burch et al. also noted that the decellularized allografts had one-tenth of the shelf life and cost substantially more than traditional cryopreserved allografts [74].

Proponents of synthetic matrices argue that customized polymers can be designed to exact specifications in a reproducible fashion. Also, by utilizing biodegradable polymers, complications associated with biocompatibility can be minimized. Initial work focused on polymers composed of PGA and PLA [75]. These "off the shelf" polymers were selected because they are biocompatible, biodegradable, well characterized, and already FDA approved for human implantation. Cells readily attach to and grow on these synthetic polymers. The resulting neotissue construct possesses adequate biomechanical properties to be used for surgical application. However, the biomechanical profile of the construct is substantially different from that of a native heart valve. TEHVs using polyglycolic acid and PLA copolymer-based matrices are thicker, stiffer, and less pliable than native valves.

Early experiments used matrices made of a PLGA woven mesh sandwiched between nonwoven PGA mesh sheets. The scaffolds were seeded with arterial myofibroblasts, followed by arterial endothelial cells and were then transplanted into the pulmonary position as a single leaflet in sheep. The seeded cells were seen in the structure after 6 weeks, and a postmortem evaluation revealed a native tissue-like architecture [76]. Furthermore, there was confirmation of elastin and collagen production in the leaflets, which had mechanical properties similar to native leaflets [76]. This approach, however, resulted in only limited success, as the PGA and PLA polymers were too stiff for use as flexible trileaflet valves.

Polyhydroxyoctanoate (PHO), a more flexible synthetic polymer has been used to create a composite heart valve scaffold in a low-pressure pulmonary system [77]. The valve consisted of a layer of PHO film sandwiched between two layers of nonwoven PGA felt. The leaflets were composed of a monolayer of porous PHO that was sutured to the conduit wall with polydioxanone. Postimplantation examination revealed a uniformly organized tissue with large amounts of collagen and proteoglycans, but with no elastin. The PHO scaffold, however, had not completely degraded after 24 weeks, suggesting a longer degradation profile than glycolic or lactic acid polymers. Additional studies revealed smooth flow surfaces but could not demonstrate a confluent endothelium which could affect the long-term durability of the structures [78]. In addition, although collagen and GAG deposition was seen, the constructs were devoid of elastin and demonstrated mild stenosis and regurgitation.

Hoerstrup et al. developed a novel composite scaffold material consisting of PGA coated with a thin layer of poly-4-hydroxybutyrate (P4HB)—a flexible, thermoplastic polymer with a more rapid degradation time than PHO [79,80]. Trileaflet heart valve scaffolds were fabricated from the composite material using a heat-application welding technique. Autologous myofibroblasts and endothelial cells from ovine carotid artery were seeded onto the scaffolds, cultured in a bioreactor for 14 days and then implanted in an ovine model [80]. When explanted, they showed increased ECM synthesis, more organized internal structure and improved mechanical properties over static controls.

It is not clear whether natural or synthetic scaffolds will ultimately prove to be more appropriate for TEHV development, as there are still certain limitations associated with both. The design of a replacement aortic valved-conduit, for example, will require the inclusion of three dilated pouches alongside the "cusp-like" leaflets to approximate the sinuses of Valsalva; AV valve designs may require the addition of other components of the AV valve apparatus, including chordae tendineae and papillary muscles [81]. Using stereolithography, Sodian et al. fabricated plastic models with an exact spatial representation of human aortic and pulmonary valves [82]. These models were then used to fabricate heart valve cell scaffolds using poly-3-hydroxyoctanoate-*co*-3-hydroxyhexanoate and P4HB, which were shown to function well in a pulsatile flow bioreactor under both normal and supranormal flow and pressure conditions. The choice and design of scaffolds will be integral to the successful clinical outcome of TEHVs. As well as conforming to a

suitable anatomical shape, heart valve scaffolds will be required to possess adequate tensile and elastic properties, being strong enough to withstand the pressure of blood flow without breaking while also not being so stiff as to impede flow. The scaffolds must be biocompatible, bioabsorbable or remodelable, and should provide a suitable template for facilitating development of new tissue. Scaffold permeability is also vital for the control of cell nutrition and removal of waste products [5].

The search for appropriate cell sources

An ideal cell source for TEHVs would demonstrate phenotypic plasticity and be able to change biomechanical properties in response to dynamic alterations in flow. It is an area of continued investigation. The use of autologous cells as opposed to xenograft or allograft tissue has the advantage of avoiding an immunological response that could result in rejection [83]. In an early attempt at tissue engineering a heart valve in 1995, valve leaflets were constructed by seeding cells from both autogeneic and allogeneic sources onto biodegradable polymeric scaffolds which were then implanted in an ovine model [76]. As anticipated, the autologous scaffold provoked less of an inflammatory response in the host, resulting in better performance and a higher success rate compared to allogeneic comparators.

A logical cell source for a TEHV would be autologous VICs and VECs harvested from a patient's own heart valve leaflet. The use of these cells would eliminate the risk of rejection while imparting the requisite phenotypic profile [5]. Preliminary studies using an ovine model utilized valve biopsy samples as a VIC source [84]. In this study, the biopsy procedure generally did not appear to compromise leaflet function, with 9 of 13 animals showing intact valves and normal leaflet anatomy postmortem. However, for human patients, it would be challenging to isolate and culture enough cells from a small biopsy to be of clinical use. It is also known that the ability to culture cells decreases with age, making this technique less feasible in older patients. In addition, patients requiring valve replacement have diseased VECs and VICs which may not be ideal for tissue engineering a replacement heart valve. Thus, the risks involved in valve biopsy are seemingly too high to make this a feasible technique in human trials. Recent studies have shown that decellularized human pulmonary valves seeded with autologous VICs and cultured in vitro demonstrated significant cell proliferation by 4 days, which suggests that such a construct can be manufactured in a shorter timeframe than initially thought [85].

Subsequent studies have utilized ovine femoral artery-derived cells as an autologous source [75]. This has failed to gain much favor given its potential for limb ischemia from disruption of the lower limb arterial blood supply. Myofibroblasts harvested from the carotid artery have also been investigated, but it was concluded by the investigators that the sacrifice of an intact tissue structure and the potential for injury introduced prohibitive risks [77]. In search of a more practical source, Shinoka et al. performed a study comparing dermal fibroblasts to arterial myofibroblasts as cells of origin for a TEHV [86]. Unfortunately, leaflets derived from dermal fibroblasts were much thicker, more contracted and less organized than those derived from arterial myofibroblasts. It was therefore surmised that cells of mesodermal origin, such as arterial myofibroblasts, provide more specialized phenotypic properties than ectodermally derived skin fibroblasts.

Myofibroblasts derived from human saphenous vein have similar phenotypic properties to VICs and represent a more realistic source for clinical applications of TEHVs [87]. Unlike arterial cells, these cells can be harvested without the risk of limb ischemia [5]. These myofibroblasts were cultured on polyurethane scaffolds and were shown to be viable and confluent at 6 weeks. Moreover, compared to neotissue derived from aortic myofibroblasts, collagen production and mechanical stability were found to be higher in saphenous vein-derived structures. Follow-up studies have compared myofibroblasts isolated from ovine tricuspid heart valve leaflets to cells from jugular vein and carotid artery [88]. Interestingly, cells from jugular veins also demonstrated higher initial collagen production, but the study showed that all cell lines had a marked drop-off in collagen, elastin and GAG synthesis with time. This finding suggests that there is a critical timeframe for seeded cells to be placed on scaffolds while they are still able to perform necessary protein synthesis and organization [59]. In the first long-term follow-up of a clinical application of a TEHV, Dohmen et al. seeded a decellularized pulmonary allograft with cells isolated from saphenous vein and cultured in a bioreactor for 2 weeks. They cite 100% survival with adequate pressure gradients and no incidence of calcification at 10 year follow-up [89].

In the search for an alternative cell source, different stem cell sources have been investigated. In particular, umbilical cord-derived cells, amniotic fluid-derived cells, and chorionic villi-derived cells appear to carry great promise [16]. One group demonstrated the possibility of using autologous umbilical cord cells [90]. The isolated cells represented a mixed population of cells derived from umbilical cord artery, vein, and the surrounding Wharton's jelly. It was found that the cells demonstrated features of myofibroblast-like differentiation, such as expression of α-smooth muscle actin, vimentin, and

deposition of collagen types I and III. More importantly, the cells successfully attached to scaffolds and formed a layered tissue-like structure comparable to scaffolds seeded with vascular cells [80]. Initial enthusiasm was tempered when it was observed that elastin was not being produced and GAGs were present only in low levels. Much more work is needed in characterization of the mixed cell population before the suitability of this source can be properly evaluated. Likewise, mesenchymal stem cells show promise, though many of the details remain to be elucidated. Their ability to develop into a variety of connective tissues, including bone, cartilage, muscle and fat, as well as easy collection via bone marrow puncture make these cells an attractive possibility [91]. In one experiment, human bone marrow stromal cells were collected and partially characterized using a number of myofibroblast markers [92]. Like umbilical cord cells, they were shown to express α-smooth muscle actin and vimentin, and produce collagen types I and III. Biodegradable polymeric scaffolds cultured with mesenchymal stem cells in vitro demonstrated an organized internal structure and mature tissue development. Despite the encouraging results, it is not clear whether bone marrow stromal cells reliably differentiate into appropriate cell types in the scaffold or if they continue to remain differentiated in vitro, ensuring long-term function and durability of the replacement heart valve [93]. Investigations utilizing circulating endothelial and smooth muscle progenitor cells are at a similar stage [94]. However, given their remarkable differentiation potential, embryonic and adult stem cells may become valuable resources for heart valve tissue engineering.

The search for an ideal replacement for both VICs and VECs is ongoing, but the underlying goal across all these techniques is the same: transplanted cell populations in a scaffold can achieve the same distribution and differentiation pattern of cells in the native valve [95]. It is hard to prove this assertion, however, as there is limited data regarding the phenotypic profile of these cells once they are implanted into the scaffolds, especially when it comes to long-term follow-up data [5].

Cell seeding techniques

Growing valvular neotissue under in vitro and in vivo conditions remains a challenge in the development of reliable and reproducible TEHVs, and much of the difficulty is rooted in challenges of cell seeding and attachment to scaffolds. Indeed, cell attachment is a critical step in initiating cell growth and neotissue development [96].

Whereas relying on adjacent autologous myocardial cell migration into unseeded valve scaffolds has proven to be an unreliable and inconsistent technique to date, directed cell seeding either in vitro or in vivo has been successful. In seeding scaffolds under in vitro conditions, early strategies for improving cellular attachment included increasing seeded cell number or density, increasing scaffold porosity, or increasing scaffold surface area. More sophisticated techniques sought to enhance cell attachment by coating the matrix prior to seeding with various cell adhesion molecules like laminin [97]. Similarly, prior to seeding a pulmonary allograft for a Ross operation, Dohmen et al. covered the decellularized valve with a commercially available synthetic ECM protein to enhance cell attachment [89]. Cell seeding was then performed using a sedimentation technique, with excellent cellular attachment and eventual neotissue ingrowth [98].

Indeed, covering valvular scaffold with molecules that can target or attract specific circulating cells offers an avenue for in vivo autologous cell seeding which precludes the need for in vitro cell culture and all the microbial risks therein. Jordan et al. utilized this principle in conjugating a decellularized porcine pulmonary valve with CD133 antibodies as a means of promoting scaffold seeding by circulating endothelial progenitor cells [99]. This strategy led to increased endothelial cell attachment, increased interstitial cell number, increased structural proteins, and improved biomechanical properties when compared with unconjugated or traditional cell-seeded valves [99].

The method of cell seeding used also influences efficiency of cellular attachment to scaffold. Traditional methods of seeding polymer scaffolds employed static cell culture techniques, in which a concentrated cell suspension is pipetted onto polymer scaffolds and left to incubate for a variable period of time, during which cells adhere to the scaffold. Dynamic cell seeding methods employ a seeding method in which either the medium or both the medium and scaffold are in constant motion during the incubation period. Dynamic cell seeding is often used in combination with a bioreactor and offers improved cellular attachment compared to static cell seeding [100]. Nasseri et al. concluded that dynamic cell seeding onto tissue engineered scaffolds increased cell adhesion, alignment in the direction of flow, cell infiltration, and seeding density [101]. There are various factors that can be further modulated in cell seeding, including use of mixed versus pure cell populations, interval cell seeding, and single-step versus sequential seeding of different cells.

Adequate, uniform and reproducible cell seeding of both natural and synthetic polymeric scaffolds remains a challenge in the field. Optimization of rapid seeding techniques will be important in the development of a TEHV, as it maximizes the use of donor cells, hastens the

proliferation and subsequent differentiation of cells, decreases the time in culture, and provides a uniform distribution of cells [102]. Advances in the modification of scaffold surfaces to enhance cell adhesion and subsequent function offers a strategy to augment ex vivo valve seeding in a timely fashion [5]. This strategy also offers the potential to direct autologous circulating cell seeding of heart valves, which if successful precludes the need for ex vivo cell culturing and allows for an "off the shelf" TEHV.

Bioreactors

The use of bioreactors for tissue engineering heart valves has increased over the last several years due to both improvement and complexity of available systems and the encouraging results that have been published with their use. Simplistically, a bioreactor is a biomimetic system used to optimize in vitro neotissue development. Ideally, the bioreactor system is designed to mimic the physiologic or pathophysiologic condition that is to be corrected with the engineered tissue. Factors such as shear stress, flow rate, flow profile, pressure, and media should be easily manipulated to change experimental conditions as researchers see fit [23]. In addition, bioreactors should be easy to access, easy to exchange media, remain sterile under long culture times, accommodate multiple samples to facilitate adequate sample sizes for statistically significant comparisons, and should be arranged to allow for direct visualization of the samples [46]. In mirroring physiologic conditions in the heart, an ideal bioreactor for tissue engineered cardiac valves consists of pulsatile flow and cyclic flex in order to generate the complex biomechanical environment that implanted valves must withstand [103]. Exposure to pulsatile flow modulates the biomechanical properties of the neotissue, which is especially important in the development of a TEHV in order to prevent premature valve deterioration [104].

One specific type of bioreactor used in the construction of a TEHV is the pulse duplicator. This pulsatile bioreactor provides physiological pressure and flow to the developing TEHV and promotes both the development of mechanical strength and the modulation of cellular function [105]. In using human umbilical cord blood-derived progenitor cells to seed heart valves made from biodegradable polymer, Sodian et al. used a pulse duplicator bioreactor wherein pulsatile flow (300−500 mL/min) and pressure (5−15 mmHg) were gradually increased over the course of 7 days [106]. This technique led to TEHVs with similar connective tissue and ECM composition as that seen in native heart valves.

Other approaches to bioreactors for TEHVs include bioreactors that can provide cyclic strain or dynamic flexural strain. Exposure of seeded valve tissue in vitro to mechanical strain results in more pronounced and organized tissue formation with superior mechanical properties over unstrained controls and results in TEHV cusps that are significantly less stiff than static controls [107]. This technique also yields substantial increases in DNA and net collagen content [108]. Cyclic flexure, which is a significant source of heart valve deformation, was used as the mechanical stimuli in a bioreactor into which cell-seeded and unseeded valves were incubated for 3 weeks. Cell-seeded valves undergoing cyclic flexure had >400% increase in effective stiffness compared to the unseeded controls and also had increased amounts of ECM [109].

Importantly, there is no uniform standard bioreactor that is used in tissue engineering heart valves, in part because of the complexities of the mechanical environment in vivo. Further understanding of the relationship between embryonic valve development and biomechanical signals may offer insight into designing an ideal bioreactor for in vitro development of heart valves. Current ongoing studies aim to take advantage of the high regenerative potential of the fetal environment as a natural bioreactor to treat congenital valve defects.

Neotissue development in tissue engineered heart valves

The process of neotissue development is poorly understood and appears to be controlled by a multitude of factors. In some ways it mimics embryonic development whereas in other ways it appears to be governed by the rules underlying tissue repair. In fact, it is likely a unique process governed by its own set of laws. The type of cells that are implanted and their interaction with the surrounding environment determine the type of tissue that ultimately develops from the cell−scaffold complex [57].

The environment in which the construct grows will influence the histological structure and ECM formed. Researchers have approached this phenomenon from several perspectives. In one approach, the formation of tissue begins in vitro by seeding cells onto a biodegradable scaffold and then maturing the tissue in a bioreactor prior to implantation in vivo. Using this approach, the scaffold is used as a cell delivery system and implanted in vivo shortly after cell attachment has taken place [58]. This approach uses a cell−scaffold construct to provide the initial structural integrity necessary to provide temporary physiological function until neotissue grows and remodels. The technique proposes that appropriate environmental signals for tissue repair and remodeling are inherently present in the in vivo milieu. In addition, in vivo biomechanical environmental forces provide

important stimuli that affect the formation of ECM and direct the biomechanical properties of the developing neotissue. The key steps in this process include [1]:

1. Cell proliferation and migration,
2. ECM production and organization,
3. Scaffold degradation, and
4. Tissue remodeling.

The mechanical and biological signals underlying neotissue formation remains an area of focused research. However, a lack of small animal models for TEHVs, which have the benefit of genetic control and large sample numbers compared to large animal models, has hindered direct mechanistic investigation into TEHV neotissue development. Much of the work in mechanisms of neotissue formation in cardiovascular tissue engineering has been done using murine and ovine models of tissue engineered vascular grafts (TEVGs), and will be discussed here. The recent development of a murine model for TEHV implantation may lead to more direct evaluations of TEHV development mechanisms in the near future [110–112].

The wide range of synthetic and natural materials used to fabricate TEHVs has led to a plethora of studies into the effects of the physical properties of the materials on neotissue formation [113]. Interestingly, recent findings suggest that microstructure morphology differences can have a profound effect on the in vivo response, in some cases being a stronger factor than the material used [114]. Utilization of a smaller fiber size was shown to reduce activation of contacting blood products [114]. Studies evaluating the effect of surface topography on platelet activation have found that a micropatterned rough surface is far less thrombogenic than a smooth surface [115]. Electrospun polytetrafluoroethylene (PTFE) demonstrated more smooth muscle cell growth and fewer adhered platelets than a flat PTFE surface [116]. In a rat aortic graft model, aligned fibers showed significantly higher patency and less thrombus formation than a graft with a smooth topography, despite similar cellular adhesion rates to both graft types [117].

Pore size has been shown to affect cell migration, with pore sizes larger than the cells encouraging migration through the scaffold, and pore sizes significantly smaller than the cells promoting cells to adhere to the surface of the graft [118]. Degradation rate is also a critical variable to consider in the development of a tissue engineered construct; a scaffold that degrades too quickly will lead to early mechanical failure, while a scaffold that degrades too slowly may lead to stiffening and inadequate neotissue formation [113]. The mismatch of the mechanical properties between an implanted graft and the surrounding vasculature has been implicated in a number of studies as a risk factor for poor outcomes [119]. Interestingly, the adjacent vasculature has also been shown to undergo significant remodeling following graft implantation, becoming stiffer and less compliant to more closely match the properties of the graft [120,121].

Recent studies of TEVGs in murine and ovine models have demonstrated that seeded mononuclear cells stimulate a robust infiltration of host macrophages during early timepoints [122]. Additional evidence suggests that circulating host bone marrow-derived cells are active in the acute inflammatory phase but do not represent a source of mature neotissue. Rather, macrophages appear to initiate an inflammatory cascade to drive vascular remodeling and neotissue ingrowth from the surrounding native vessel [123]. Other pathways specific to heart valve physiology may also be vital to understanding neotissue formation. One key process in the development of the heart valve is EMT. It has been demonstrated that EMT pathways involving TGF-β3, BMP2 and VEGFA are activated and localized in TEHV remodeling [124]. Many groups believe that the identification of biomaterials that can induce EMT and direct cellularization of TEHVs will be invaluable to the field [59].

TEHVs have been evaluated biochemically, molecularly, histologically, physiologically, biomechanically, and morphologically (Fig. 34.3). In each type of analysis, the tissue engineered valve has been compared with the native valve [93]. These data can then be used to identify shortcomings in the TEHV and aid in the rational design of an improved version. One challenge that remains is the identification of good biomarkers or methodologies for the noninvasive study of the structural remodeling and functionality of in vivo constructs so that a tissue engineered valve can be followed over time [125].

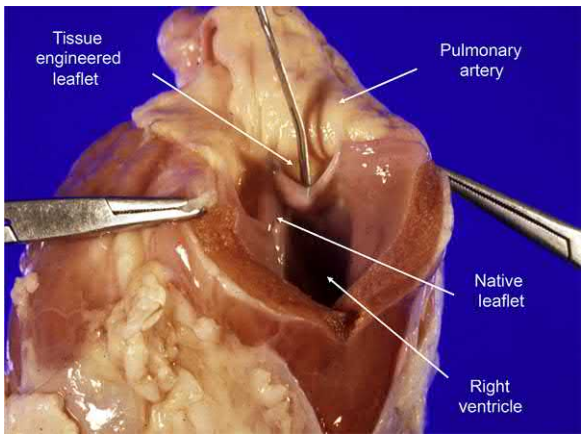

FIGURE 34.3 Photograph of a tissue engineered valve in a sheep heart.

Clinical applications of the tissue engineered heart valve

Dohmen et al. reported the first successful use of a TEHV using a decellularized cryopreserved pulmonary allograft [89]. Since then, Dohmen et al. have published long-term data on a series of 11 patients who underwent the Ross operation with a TEHV to surgically reconstruct the right ventricular outflow tract. In each case, the allograft was seeded in a bioreactor with autologous vascular endothelial cells that had been isolated from a segment of forearm vein 2–4 weeks prior to the operation. At 10 years follow-up, the TEHVs showed excellent hemodynamic function. All patients remained in New York Heart Association class I heart failure and computed tomography showed no evidence of calcification or valve degeneration (Fig. 34.4) [126]. Despite the lack of a control group, the small sample size, and the lack of long-term biochemical or tissue samples, this study is a significant advance in the clinical application of TEHVs. Re-analysis of the group of patients at 15 and 20 years postimplantation will offer insight into the effect that autologous cell seeding has on allografts in preventing long-term complications that are seen in unseeded allografts and homografts, such as calcification and valve degeneration.

Other investigators have used decellularized scaffold implantations with autologous cell seeding to tissue engineer cardiac valves. While this technique does not include any ex vivo cell seeding typical of tissue engineering approaches, this method holds the promise of valve implantation of allogenic or xenogenic tissue with a decreased host response to donor antigens. In lieu of data that host antigen recognition and antibody development may be linked to early tissue calcification and degeneration, the advantage of decellularizing a scaffold prior to implantation is that the cellular antigen burden decreases while the extracellular architecture and material properties are maintained [127].

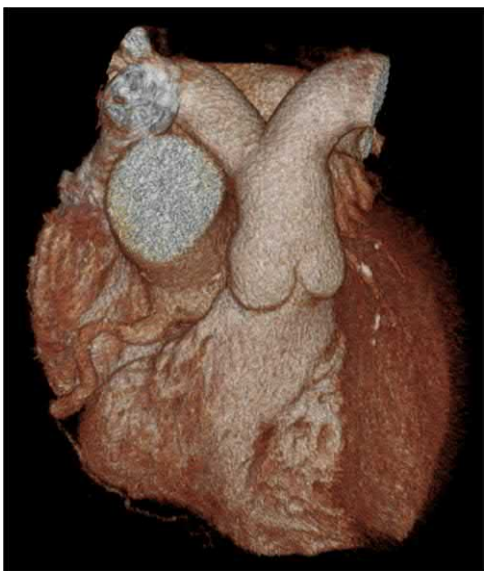

FIGURE 34.4 Multislice computed tomography shows a normal tissue engineered pulmonary valve, without evidence of calcification or degeneration after 10 years in a male patient who was operated on at age 46 [126].

Use of xenografts in this fashion was halted in 2003 after Simon et al. reported failure of SynerGraft decellularized porcine valves implanted in four children as pulmonary valves for right ventricular outflow tract reconstruction. In this study, two valves were severely degenerated at 6 weeks and 1 year after implantation, and one valve ruptured 7 days after implantation, ultimately resulting in three deaths and the fourth child's valve was explanted prophylactically [73]. Since then, focus has turned to allogenic decellularized pulmonary valves using the SynerGraft process with results so far showing promise for this technique over standard cryopreserved allogenic valves. Studies published to date comparing SynerGraft decellularized allogenic valves with standard cryopreserved valves show in the SynerGraft valves decreased short-term stenosis or regurgitation, decreased clinically significant insufficiency, lower peak valve gradients, and fewer interventions [128]. Brown and colleagues performed a multicenter retrospective cohort study of 342 patients undergoing right ventricular outflow tract reconstruction with SynerGraft or standard cryopreserved valves and showed decreased regurgitation in the SynerGraft group and overall safety and efficacy at 4 years postimplantation [52].

In contrast to the enzymatic decellularization technique utilized by Synergraft, the Matrix P and Matrix P Plus xenografts were decellularized using only detergents, including Triton X-100, sodium dodecyl sulfate, and sodium deoxycholate, in the presence of protease inhibitors [129]. Early studies with Matrix P and Matrix P Plus in right ventricular outflow tract reconstruction, using TEHVs made from decellularized porcine pulmonary valves, demonstrated 87% freedom from intervention at 1 and 3 years postoperatively [129]. However, further studies showed a freedom from failure and dysfunction rates of 60% and 77% at 2 years and less than 20% freedom from failure at 3 years, with the most common cause for failure being conduit stenosis. Histological samples of explanted valves revealed poor cell growth into the scaffold and an abundance of inflammatory giant cells. Age less than 1 year at implant was associated with poorer outcome [130]. Further studies showed the grafts elicited a strong foreign body reaction of innate and adaptive immune cells, as well as a low neovascularization and high fibrotic deposition. These studies also showed that the grafts may have been incompletely decellularized, increasing their immunogenicity [131].

The complications of several early clinical trials with TEHVs have led to a resurgence of large animal research into TEHV neotissue development using a wide range of candidate materials, including decellularized valves, porcine small intestinal submucosa, and a PC-BU polymer seeded with a fibrin gel [132–134]. As these studies continue to provide more insight into the mechanisms of successful valve development and possible failure modes, there is hope for a new wave of TEHVs to begin to approach the clinic.

To date, synthetic scaffolds have not been used to fabricate a replacement heart valve for clinical use. However, the principles of tissue engineering outlined thus far and the approach of identifying and isolating a cell source, choosing a scaffold, and seeding the cells on to the scaffold were applied by Shinoka et al. in the first human clinical trial investigating TEVGs in children with complex congenital heart disease. The study began in 2001 and included 25 patients with an average age of 5 years undergoing extracardiac total cavopulmonary connection using the TEVG as a conduit. The synthetic scaffold used was a copolymer of polyglycolic acid and ε-caprolactone reinforced with poly-L-lactide. The scaffolds were seeded with autologous bone marrow-derived mononuclear cells. At 1 month postimplantation, all patients were alive and symptom free, and radiographic studies demonstrated no cases of TEVG aneurismal dilatation, thrombosis, or stenosis. Patients were followed serially with angiography, computerized tomography, or magnetic resonance imaging examinations. At 1 year there was one patient diagnosed with partial mural thrombus who was treated successfully with anticoagulation [135]. At mid-term follow-up (average 5.8 years postimplantation), four patients had died of causes unrelated to the TEVG and four patients had developed TEVG stenosis requiring balloon angioplasty or stenting [136]. At a late-term follow-up (average 11.1 years postimplantation) there were still no cases of graft related mortality in any of the 25 patients. In total, seven of the twenty-five patents (28%) developed asymptomatic stenosis that was treated with angioplasty [137]. Overall, the tissue engineered vascular conduits had reduced incidence of calcification, no risk of rejection because of autologous cell seeding, minimal risk of infection, and potential for growth [138]. Following the excellent results of Shinoka et al., the first FDA-approved human clinical trial investigating use of TEVGs in children with congenital heart disease was recently completed. Shinoka's results prove that the application of tissue engineering principles to vexing clinical problems has the potential to dramatically improve outcomes and lessen the morbidity and mortality of disease. All of these potential benefits would hold true for a TEHV.

As the field of cardiovascular tissue engineering continues to expand, so too will the desire to apply new devices, methods, and models in human clinical trials. It is essential for investigators to have thoroughly established the efficacy of techniques in large animal and preclinical models prior to translation into humans. Issues of bacterial contamination, insufficient cell seeding, and rapid degeneration of scaffold are common complications of tissue engineered devices and therefore strict protocols must be in place and adhered to in order for this promising field to expand clinically in an effective and responsible manner. While randomized clinical trials are the gold standard, it is not always possible to utilize this trial design, particularly for orphan applications such as TEHVs. Most importantly, though, for physicians, scientists, regulators, and engineers, is to apply this promising technology in a responsible manner, placing the safety and well-being of the patients as the top priority in all instances.

Conclusion and future directions

Successful development of a tissue engineered replacement heart valve holds the key to better treatment and improved clinical outcomes for end-stage valvular disease. Although significant progress has been achieved since its inception in the early 1990s, the field is young and many key issues have yet to be resolved. We are still exploring the cellular and ECM biology that govern the maintenance of a normal valve. Better characterization of valve cells like VECs and VICs may offer clues to optimize cell seeding. Recently developed small animal models for heart valve implantation may lead to deeper understandings of the mechanisms involved in valve repair and the formation of neotissue into a functioning valve. Moreover, advances in other fields of tissue engineering and stem cell biology may provide new cell types and processing strategies that could transform either the cell source or cell seeding technique used in TEHVs.

While still in its infancy, there has been some investigation into the use of TEHVs in fetuses at risk for hypoplastic heart syndromes. Hypoplastic heart syndromes are believed to arise because of poor intracardiac blood flow in-utero. Severe aortic stenosis leads to left ventricular dilation, dysfunction, and ultimately left ventricular growth arrest [139–142]. In fetuses with aortic stenosis, aortic valvuloplasty has been shown to restore flow and enable postnatal biventricular circulation in patients who otherwise have a high likelihood of developing hypoplastic left heart syndrome [143,144]. After birth, however, patients treated with valvuloplasty are at risk for aortic regurgitation and may require additional procedures such as valve replacement, which the neonate is likely to

outgrow several times during his or her development. A preferable paradigm would be to utilize tissue engineering principles to prevent the progression of ventricular growth arrest to allow for biventricular circulation, while simultaneously providing a competent TEHV that grows with the fetus.

The success of transcatheter heart valve implantation is well-established, and fetal aortic valvuloplasty has recently reached use in the clinic. Although some investigators have begun establishing animal models, there is little existing literature on the combination of these techniques towards transcatheter implantation of stents and heart valves in fetuses. Weber et al used a hybrid approach in which pregnant ewes underwent a midline laparotomy, followed by fetal heart cannulation and guidewire placement through the ductus arteriosus into the aorta. A 14-French delivery system was then advanced to the pulmonary artery where a stent was successfully deployed [145]. This demonstrated the feasibility of prenatal stent delivery into the pulmonary artery using a hybrid trans-apical technique in a sheep model. Our group's preliminary experience with endovascular interventions in fetal lambs suggests that percutaneous delivery is feasible. In ongoing preclinical work, we have successfully percutaneously implanted TEHVs in the right ventricular outflow tract of fetal sheep, using a 17-gauge trocar needle introduced through the maternal abdomen. The work thus far is promising and the prospect of introducing TEHVs into the fetal milieu holds considerable promise for the clinic, as well as innovating ways to harness fetal development for tissue engineering.

Development of surgical techniques for valve implantation in the fetus may allow for better outcomes, as the fetal milieu is known to have a higher regeneration potential. Transcatheter valve deployment approaches have the benefit of being much less invasive than standard open surgical techniques, allowing for more intact native environment for tissue engineered valve remodeling. Traditional stents do not degrade over time, limiting their usefulness in tissue engineering approaches. However, recent developments and research into degradable stents may allow transcatheter deployment of tissue engineered valves. Similarly, growth in other fields like 3D printing or quantification of flow using magnetic resonance imaging may eventually find clinical applications in the technologic advancement of TEHVs. To that point, it is clear that tissue engineering is a multidisciplinary, multifaceted field that requires cooperation, coordination, and collaboration between experts in a variety of different specialties. Fostering these types of relationships using unique funding mechanisms and programs will help move this field forward and will ultimately benefit the patients that depend upon its growth.

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Part Ten

Endocrinology and metabolism

Chapter 35

Generation of pancreatic islets from stem cells

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Introduction

Diabetes mellitus caused by the destruction of β-cell through autoimmune-mediate attack has become the most serious, chronic, and expensive disease. The related glucose homeostasis disruption leads to acute complications or death if left untreated. As the only available treatment for diabetes consist of exogenous insulin supply and while islet transplantation temporarily confers normoglycemia to patients, the lack of a renewable source of insulin-producing β-cells hampers the use of this treatment option. Although significant hurdles remain, in the last decade, tremendous progress has been made in generating insulin-producing cells from both mouse and human pluripotent stem cells. Following the principles that guide pancreas embryonic development is a common aspect in all differentiation protocols with considerable success in generating β-like cells in vitro. Greatest outcome of the refined protocols became apparent in the first clinical trial announced by the ViaCyte, Inc. in October 2014 and recently in Europe by the Center for Beta Cell Therapy in Diabetes (Brussels) in January 2019, based on the implantation of pancreatic progenitors (PPs) that would further mature into functional insulin-producing cells inside the patient's body. In this chapter, we will update and discuss the state-of-the-art in β-cell−replacement therapies based on the differentiation of pluripotent stem cells into glucose-response and insulin-producing cells of potential use in the treatment of type I diabetes.

State-of-the-art

A first breakthrough for in vitro differentiation and selection strategies was reported by Soria et al., when it was showed for the first time that the process was doable [1]. Combination of directed differentiation with gene-trapping strategies succeeded in the manufacture of insulin-producing cells derived from rodent embryonic stem cells that normalize blood when implanted into streptozotocin diabetic mice [1]. Further reports improved the system introducing new differentiation strategies, such as inhibiting sonic-hedgehog, or selecting cells that express a gene also expressed in islet progenitor cells (Nkx6.1) [2]. It was also shown that differentiated cells do not form teratomas [2,3], mature after 30 days inside the host [2], and effect on animal models in dose-dependent [4]. A definitive breakthrough was the demonstration that the process may be translated into human cells [5−8]. In vitro differentiation protocols for human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs) succeeded in generating definitive endoderm by using Activin A [5,6,8]. From then a tremendous list of protocols has been published that ends with insulin-producing cells derived from pluripotent stem cells. The limited space of this commentary does not allow acknowledging the full amount of contributions. However, in vitro differentiation protocols reached a non-return outcome in October 29, 2014 when the first clinical trial was announced by ViaCyte, Inc., San Diego, CA [9]. The strategy of ViaCyte, Inc. consists in implanting β-cell progenitors subcutaneously into a biocompatible capsule that may eventually be removed. The pilot study (ClinicalTrials.gov Identifier: NCT02239354) approved by the Food and Drug Administration is currently recruiting participants; however, no information has been made public yet on the evolution of the implanted patients.

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After the proof of concept was established in rodent embryonic stem cells (ESCs) [1] and hESC [5–8], more than 100 papers reported the conversion of different stem cells and progenitors into the insulin-producing cells. Here we will discuss the consolidated knowledge using mammalian embryonic stem cells as well as innovative concept of islet cell regeneration giving support to a new era in the treatment of diabetes mellitus.

The current success in generating pancreatic cell lineages from hESCs relies on recapitulating the key events that regulate pancreatic lineage commitment in the embryo. The advances in our understanding of the key transcription factors and signaling pathways that govern pancreas development and β-cell formation have been crucial for the design of new protocols for generation of in vitro insulin-producing cells from ESCs. Our knowledge of human pancreas development is derived largely from models of animal, such as rats, chicks, fish, and mainly mice, and it assumes that the molecular and cellular aspects of pancreas development are conserved, although some aspects of the mouse ESCs differentiation protocols may differ from those applied to hESCs differentiation protocols.

The challenge of making a β-cell

Tremendous progress has been made in the field of pancreas development, and it has been extensively reviewed [10,11]. Here, we will focus on the main transcription factors that govern different stages of islet development and β-cell fate; thus the common approach to differentiate hESC and human-induced pluripotent cells toward β-cells is based on a multistaged protocol attempting to reproduce in vivo pancreas development. Protocols aim to induce hESCs and hiPSCs to follow a sequential transition through mesendoderm, definitive endoderm, gut-tube endoderm, pancreatic endoderm, and endocrine precursor stages, to finally obtaining functional insulin-expressing cells [12–16]. Reported signaling pathways and factors required to direct pluripotent stem cells differentiation toward functional insulin-secreting cells are the result of years of investigation. However, due to the complexity of the aim, a highly efficient differentiation protocol is still missing.

Recent reports have detailed strategies that overcome prior hurdles to generate β-like cells from hESC and hiPSCs cells in vitro [12,13]. As the field progresses toward generating therapeutic β-cells from stem cell precursors, there are several hurdles to overcome. First, the cells must sense glucose and respond with appropriate insulin secretion. Over the last 10 years, improved protocols to direct the differentiation of hESCs into the pancreatic lineage have been established using combinations of small cell permeable molecules (growth and differentiation factors), such as retinoic acid, kinase C activator (Indolactam V, PdBU), fibroblast growth factors (bFGF), TGFb pathway inhibitors (Alk5 inhibitor, Dorsomorphin, or Noggin), BMP inhibitors, Sonic hedgehog pathway inhibitors (KAAD-cyclopamine or SANT-1), nicotinamide, thyroid hormone, nitric oxide (NO), and resveratrol or miRNAs (miR-7) [14–19]. However, in a typical multistage protocol, the final cell population has about 20%–30% stem cell–derived β-cells. Therefore improving the efficiency remains an important challenge. The necessity to obtain mono-hormonal insulin (C-peptide)-positive cells with high efficiency has led, in addition, to the identification of new molecules that not only triggered maturation of pancreatic endocrine precursors toward a β-cell phenotype but also improved glucose-stimulated insulin secretion in differentiated β-cells. Kieffer's group described a seven-stage in vitro differentiation protocol that efficiently converts hESCs into insulin-producing cells. Their so-called Stage-7 cells expressed key markers of mature pancreatic β-cells and also displayed glucose-stimulated insulin secretion similar to that of human islets of β-cells during static incubations in vitro [13]. Most recently, this approach reached an outstanding challenge by showing that upon transplantation into immunocompromised mice, the cell transplanted restored glycemia within months [3] or weeks [12,13] of the transplantation. Something that seems to depend on the state of maturation of the cells, since one of the great dilemmas is the full cell maturation that may be possible in vitro or may occur shortly after transplantation. In addition, the protocols take a huge economic and time commitment that is nontrivial.

Recent achievements (first generation of pancreatic progenitors used in the clinic)

The first pioneering study focused on hESC to make PP cells that can turn into insulin-producing cells, providing the first compelling evidence that implantation of hESC-derived pancreatic cells into mice resulted in the generation of glucose-responsive insulin producing cells. These results laid the foundations for the beginning of a prospective, multicenter, open-label, first-in-human phase 1/2 clinical trial (NCT02239354) in 2014, conducted by ViaCyte, Inc. to deliver the PP cells in an immunoprotective device product, PEC-Encap (also known as VC-01), in subjects with T1D. Next, in 2017, a clinical trial was started (NCT03163511), testing ViaCyte's PEC-Direct product (also known as VC-02), a new open device allowing direct vascularization of stem cell–derived PEC-01 PP cells, but that requires immunosuppressant drugs, and recently, in January 21, 2019, the Center for Beta Cell Therapy in Diabetes, coordinator of an international consortium in translational medicine in diabetes, and

ViaCyte, Inc. announced that VC-02 has been implanted in type 1 diabetes patients at a subtherapeutic dose. These are the first patients in Europe to receive PEC-Direct, an encapsulated PP cell product candidate designed to replace lost insulin-producing β-cells and restore blood glucose control for type 1 diabetes patients who fulfill entry criteria for a β-cell replacement therapy. Therefore the long-term function of these cells in vivo is still unclear, highlighting the presence of survival issues of encapsulated cells in human. In this scenario, to keep the graft viable in long-term patients after cell transplantation further strategies that improve both cell retention and long-term cell viability should be implemented. Their longevity in vitro and in vivo has not been clearly studied. To achieve a high ratio of survival and glucose response of transplanted β-like cells in vivo, several major issues should be considered, such as, determining an optimal transplant site in the body [20,21], accelerating maturation of transplanted β-like cells in vivo [22–24], protecting transplanted β-like cells from immune attack and inflammatory reactions in the patient [25–27], and facilitating the vascularization of the transplanted β-like cells and the release of insulin [28]. Besides, reliable assays are critically needed to monitor graft potency in hESC-derived pancreatic cells transplantation. These cells must show many of the key features and functional similarities to human β-cells in terms of gene expression [29,30], insulin content [1], glucose-stimulated human c-peptide/insulin secretion [12], ultrastructure of insulin granules [12], and glycemia modulation in streptozotocin (STZ)-induced diabetes models [3,12,13]. Several methods have been proposed to monitor qualitative or quantitative characteristics of hESC-derived PP. Global gene expression analysis is used to assay the expression of several endocrine cell markers for the initial evaluation of hESC differentiation capacity. Clusters with PDX1 + /NKX6-1 + cells can be transplanted into mice to generate glucose-responsive cells in vitro and in vivo.

Need of late maturation: cabimer protocol

Over the past decade, much has been published about the differentiation protocols for the generation of insulin-producing cells from hESCs and/or hiPSCs, in the pursuit for the recipe to make the best insulin-producing cells. Although essential growth and differentiation factors have been identified, an appropriate response to glucose in terms of insulin release was not obtained until the cells were transplanted, when subsequently matured into functional β-like cells in vivo.

Several groups are working to refine the recipe [12,13,31]. As a result, refined protocols are being elaborated to turn immature pancreatic endocrine precursors into functionally mature glucose-responsive β-cells before transplantation. In line with this proposal, different factors, that influence the maturation, enable glucose-stimulated insulin secretion in vitro, and obviate the need for in vivo "maturation" to achieve functionality, were proposed. Among various tested compounds a few small molecules, stimulating sirtuin activity, such as resveratrol, that has been demonstrated improves glucose homeostasis, decreases insulin resistance, protects pancreatic β-cells, improves insulin secretion, and ameliorates metabolic disorders [32]. Therefore this function can be harnessed in the later stages of β-cell differentiation in vitro. Resveratrol yields functional and transplantable insulin-producing cells capable of restoring glucose homeostasis diabetic mice [14]. Recent evidence reveals that miRNAs acts as switch in the maturation of insulin-producing cells by regulating the expression of targets to affect insulin synthesis and secretion [33]. A number of miRNAs are important regulators of β-cell differentiation and function, including miR-375 [34], miR-24, miR-148 [35], miR-200, miR-30d, miR-124a [36], miR-9, miR-15a, miR-16, miR-146a, miR-29a, miR-34 [37,38], and miR-7, the latter being a key regulator to regulate functional aspects of glucose-stimulated insulin secretion [15]. Therefore miRNAs involved in the formation of hESC-derived pancreatic cells, should be considered for improvement in the regulation of insulin synthesis and secretion. These studies reveal that metabolic maturation could be conducted in vitro without the need for in vivo maturation. Efficient assays for control points at each pancreatic development stage are essential to characterize the transcripts that are expressed in a stem cell–derived pancreatic β-like cell. While insulin is the "sine qua non" of the β-cell, insulin expression alone does not suffice to convey mature β-cell identity. Additional traits in the resulting cell population such as, glucose-sensing, cell excitability, insulin synthesis, packaging, and secretion, are required to transform a mere insulin-expressing cell into a mature and functional β-cell capable to release insulin in a pulsatile fashion [39]. A network of transcription factors underlies the regulation of a number of genes required for these functional traits, including PC1/3, NKX6.1, PDX1, PAX4, NKX2.2, NEUROD, PAX6 ISL1, and MAFA specific markers of β-cell lineage [40–46] (Fig. 35.1).

Strategies to maintain cell viability

One of the main challenges affording cell therapy for patients with diabetes consist of finding the way to enhance the ability of transplanted cells to integrate into tissues, to survive, and to induce healing and growth. Securing an optimized in vitro differentiation protocol, the field must now turn toward how to address

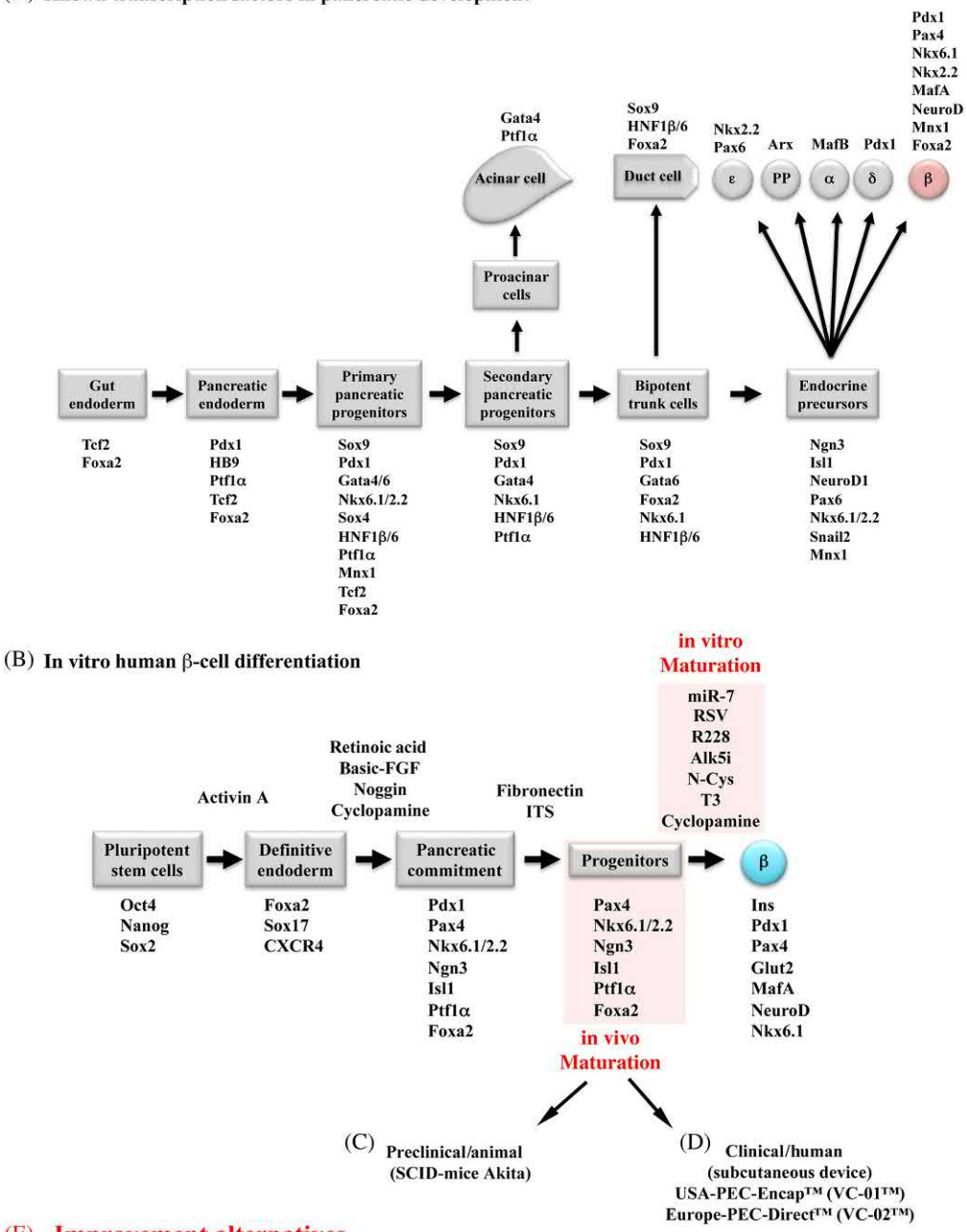

FIGURE 35.1 Simplified model of pancreas organogenesis, differentiation and maturation strategies, and alternatives to obtain a functional β-cell. (A) Transcription factors required during the development of the islets of Langerhans. (B) Sequential expression and temporal variation of genes during in vitro differentiation protocols. (C) Pancreatic progenitors to specialize into functional β-cells through maturation after transplantation into STZ-induced hyperglycemic mice. (D) Strategy for subcutaneous implantation of encapsulation devices with β-cell progenitors that can mature inside the patient's body and, eventually, control blood glucose and protect from immune insult. (E) Strategies to be considered to improve the process. *Adapted from Soria B, Gauthier BR, Martín F, Tejedo JR, Bedoya FJ, Rojas A, et al. Using stem cells to produce insulin. Expert Opin Biol Ther 2015;15(10):1469–89.*

immunological barriers for transplantation? And thus, to deal with immunosuppression, encapsulation, cell delivery, and to take advantage of gene editing. The first possibility for the allogenic transplant requires lifelong immunosuppression to control both alloimmunity and autoimmunity and to prevent the rejection of the graft [47], although the possibility of using a patient-specific induced pluripotent stem cells (iPSC)-based personalized-medicine strongly arise [48]. In addition, the immunosuppressive regimen required for pancreatic stem cell−replacement affect at the same time the proliferation and survival of the transplanted cells [49]. Though, the best way to improve cell survival is to build an artificial niche that mimics the extracellular matrix in vivo, from this starting point, one can add additional features, such as molecular signals, that enhance cell resistance or structures that protect cells from host's hostile. Therefore the β-like cells must be protected and isolated by a physical barrier to escape the immune system attack. Still, the long-term viability and functionality of the cells depend on the physiological microenvironment conditions of the host. Transplantation of β-like cells into humans must also involve encapsulation technology, not only to prevent destruction of the grafted cells by the immune system but also to contain the graft itself [50]. Examples of extravascular macrocapsule technologies, that display promise for PP cell transplantation, includes the TheraCyte and ViaCyte implant systems [25−27] and the polymer encapsulated with alginate derivatives [51]. So far, no approach has succeeded to prevent immunologic rejection of cells. A recent strategy to evade the immune rejection is based on the possibility of genetically modified cells to evade the immune system. CRISPR gene editing has the potential to protect the transplanted cells from the patient's immune system via ex vivo editing of immune-modulatory genes within the stem cell line that is used to generate the pancreatic-lineage cells. In this regard, CRISPR-Cas9 genome editing is used to specifically switch off antigenic determinants of the cell surface and therefore to escape postimplantation rejection [52−54].

Encapsulation and tolerogenic strategies

The next major challenge is the difficulty in maintaining graft functionality in spite of immune rejection by recipients. Cellular encapsulation technologies are an attractive strategy to protect transplanted β-like cells from immune attack, being able to prevent both allogeneic rejection and autoimmune attack. Encapsulation strategies are based on embedding insulin producing cells in solid matrices, creating a semipermeable environment around β-cell-like cells capable of immune-protection, but also allowing for efficient mass and oxygen transfer [50,55,56]. Based on published records, we can affirm that mature pancreatic cells delivered in a cell encapsulated−based systems accelerate the development of glucose-sensitive for β-like cells and warrant further investigation, given that, successful development of cell therapies will require consideration of processes controlling both β-cell development and mature function [15,57]; success of these cell strategies also include how amenable the protocols are to scaling-up in order to generate the hundreds of millions of cells required per patient [58,59], and encapsulation of β-like cells has demonstrated efficacy long-term control of blood glucose levels with a single minimally-invasive outpatient implant [60,61]. These approaches face challenges, including oxygen and nutrients limitations, due to the lack of vascularization and the possibility of a fibrosis coating the graft as a foreign material [62]. Vascularization is the major factor that determines whether the transplanted graft can survive and respond to the glucose fluctuations in vivo. Both healing and vascularization around the grafts are considered as important variables; a previous vascularizing pretreatment at the implant site may be essential for success in the correction of hyperglycemia through its increases and/or maintains cell survival, endocrine differentiation, and/or β-cell maturation [28,63]. Innovative biomaterial coatings and novel encapsulation design are being developed to overcome these challenges toward long-term cell function or survival after transplantation and to enable clinical success and minimize risks of encapsulated β-like cells or islets.

The concept of cellular medicament

Regulatory authorities, such as the US Food and Drug Administration (United States), the Public Health Agency of Canada (Canada) and the European Medicines Agency (Europe), established that when cells are used to treat diseases, they have to be considered "cellular medicaments" and fulfill the same criteria of small molecules and biologicals [64]. So far, the only established cell therapy is bone marrow transplantation. Cell therapy of diabetes is still an experimental "cellular medicament" and has to follow the well-established pattern of Phase I (safety and viability), II (efficacy), and III (efficiency). Before authorization of use and possible commercialization, the dossier has to be approved by the Regulatory Agencies. Our impression is that there is still room to improve these cellular medicaments by iterative preclinical and clinical research. Since we are aware of the high cost of this development and that both ViaCyte, Inc. and BetaLogics are subsidiaries of Janssen Pharmaceutical (Johnson and Johnson group), we wonder whether these medicaments will be cost-effective and affordable for the millions of people that suffer diabetes.

Conclusion

Despite the positive outlook for the future of stem cell–derived treatments for insulin-dependent diabetes, there is still two major challenges to overcome, the first is the need for better control over the methods that make β-cells from stem cells and the second is preventing an autoimmune attack after transplanting the stem cell–derived β-cells into patients. In this scenario, further studies are required to generate new transplantable insulin-producing cells that are safe and able to mimic closely the complex functions of an endogenous β-cell and to overcome several hurdles; the list is too long, but we summarize the following to be solved: in vitro differentiation strategies to generate either mature postmitotic β-cells or β-cell progenitors that may be safely implanted into the host; cell-selection methods to end with a pure β-cell population; election of the best encapsulation device to implant the cells that favors oxygen supply, promotes neoangiogenesis, and displays tunable injectability, porosity, stiffness, avoiding the foreign body reaction, and mimicking the extracellular matrix natural microenvironment; elucidation of mechanisms underpinning the therapeutic effects in preclinical representative animal models; and validation of cell–host interactions. Besides, given the enormous task that we are facing, we also suggest that a private–public international consortium with transparent rules may be a more efficient way to reach a safe, effective, and affordable cure for diabetic patients.

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Chapter 36

Bioartificial pancreas: challenges and progress

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Introduction

Type I diabetic (T1D) patients have lost the majority of their insulin-producing cells due to an autoimmune disease [1]. As a consequence, the patients depend on daily insulin injections for their regulation of glucose levels. Although this therapy is lifesaving, it does not regulate glucose-metabolism as tightly as the endocrine pancreas. Patients may therefore still suffer not only from diabetic complications such as nephropathy and retinopathy but also from vascular issues responsible for cardiac and brain damage [2–5]. The chances of these complications do decrease when glucose levels are intensively regulated by regular measurements of glucose levels and injection of insulin according to the patients' needs, but this therapy is associated with regular hypoglycemia and disabling hypoglycemic unawareness [3,4].

A better therapy is highly needed in order to improve the quality of life of T1D patients. An option is to transplant the endocrine pancreas [2]. Basically, there are two options: transplantation of the whole pancreas or only the endocrine pancreas. Transplantation of the whole pancreas is done in specialized centers in patients that as the consequence of end-stage renal failure receive a kidney transplant [6,7]. Although the grafts are in most cases successful in inducing normoglycemia and insulin independence, the procedure is associated with major surgery, some morbidity and severe complications may occur [7]. This makes the procedure limited to those patients that suffer from end-stage diabetic complications. Transplantation of only the endocrine pancreas, that is, the insulin-producing pancreatic islets is not associated with major surgery as it involves not more than infusion of tissue via the portal vein and in principle can be applied in a far larger group of T1D than patients that currently qualify for whole pancreas transplantation.

Transplantation of only the endocrine pancreas experienced a major breakthrough in 2000 with the introduction of the so-called Edmonton protocol that involved the introduction of immunosuppressive protocols lacking steroids [8,9]. With this protocol the majority of the T1D recipients of an islet graft became normoglycemic and insulin independent. Most patients remained insulin independent for more than a year, but after 2 years, 50% of the patients were still insulin independent [10]. The 50% that became insulin dependent still experienced improvement in quality of life as hypoglycemic events or unawareness were virtually absent in this group compared to the pre–transplant period. The technique has further evolved in the past two decades. Some experienced groups are able to successfully transplant islets from one single donor by controlling the parameters known to influence the islet yield [11,12].

Although important advances have been made in clinical islet transplantation programs, the procedure still requires lifelong administration of immunosuppressive medication. This is a major obstacle for wide application in T1D patients as immunosuppressive protocols have serious side effects in recipients [13,14]. It is unlikely that immunosuppression will ever be an acceptable alternative for insulin therapy in T1D. This is the main reason why the scientific community is searching for approaches to allow transplantation of islets in the absence of chronic immunosuppression. This is how the bioartificial pancreas was introduced. By packing insulin-producing cells in semipermeable but immunoprotective membranes islets

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can be protected from the host immune system while regulating glucose metabolism on a minute-to-minute basis.

History of the bioartificial pancreas

The development of encapsulation technologies to immunoprotect cells has a long history and dates back to 1933 [15]. In the groundbreaking publication of Bisceglie [15], tumor cells were encapsulated and implanted in the peritoneal cavity of pigs to follow the fate of the cells when free floating in the device in the absence of vascularization (Fig. 36.1). Bisceglie [15] applied amnion tissue to encapsulate the cells. Already at that time it was recognized that these tissues have semipermeable properties and some degree of immunoprotection. The authors demonstrated prolonged survival of the encapsulated tissue and therewith introduced the concept of cell-encapsulation for prevention of graft rejection. However, it took till 1950 when Algire et al. [16] recognized the potential of the technology for the cure of endocrine diseases. They [16] created artificial polymeric diffusion chambers in which therapeutic cells were encapsulated with the aim to create an immunoprotected microfactory involving cells that release therapeutics upon demand. The proof of principle was demonstrated but Algire et al. [16] also demonstrated the importance of application of fully biocompatible materials and the need for defining permeability properties. Since the 1980s numerous devices have been published in different conformations with applications of many different polymeric biomaterials of different compositions. It has let to testing of the devices in many disorders where management of the disease needs a minute-to-minute regulation of metabolism such as in hemophilia B [17], anemia [18], dwarfism [19], kidney [20] and liver failure [21], pituitary disorders [22], central nervous system insufficiency [23], and diabetes mellitus [24]. In the past two decades, important advances have been made in the technology of cell-encapsulation. Many of those studies focus on application in T1D as the disease is affecting 1.25 million individuals in the United States alone and is associated with $9.8 billion on health care cost [25]. These costs can be heavily reduced if a therapy is developed that tightly regulates glucose levels. Encapsulation of cells is considered to be such an approach.

Replenishable cell sources and encapsulation

During the past 5 years, encapsulation technologies have received much attention by the scientific community. One of the leading reasons for this is the advances in replenishable insulin-producing cell sources (Fig. 36.2). In principle, these cell sources provide an inexhaustible source for insulin-producing cells for the large group of T1D patients. As most encapsulated grafts still demonstrate limited survival times such a replenishable cell source may also allow replacement of the graft after cease of function, which also may facilitate application of the technology in a wider group of patients.

Most of the replenishable cell sources are of allogeneic or xenogeneic origin and require an encapsulation technology to prevent rejection of the cells. There are several reports demonstrating the usefulness of encapsulation for immunoprotection of replenishable cell sources. Pagliuca et al. [26] developed glucose-responsive stem cell–derived β cells that in another study were encapsulated in alginate-based microcapsules and were implanted in T1D mice models, which induced normoglycemia for up to 174 days [27]. The protocols for maturation of human stem cell–derived β cells has

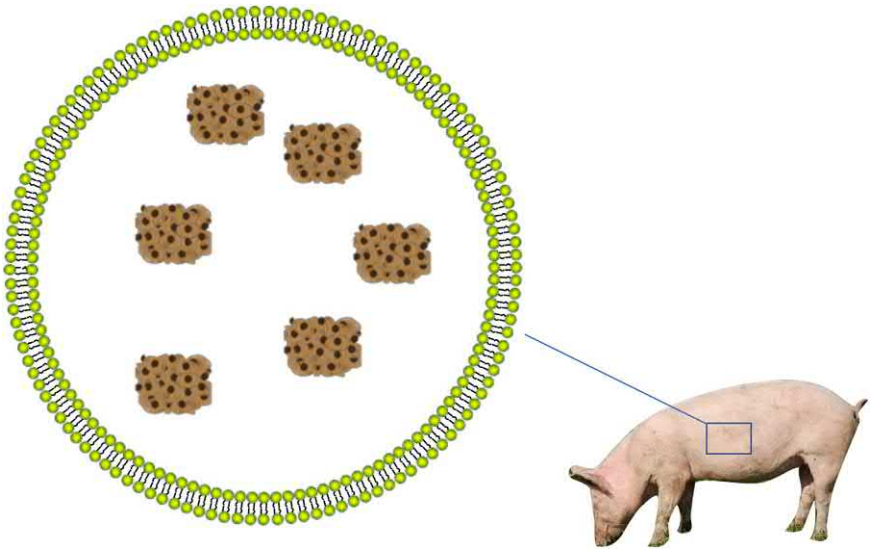

FIGURE 36.1 The concept of cell-encapsulation for immunoprotection was introduced as far back as in 1933. Bisceglie [15] implanted tumor cells after encapsulation in an amniotic sac into the peritoneal cavity of pigs to study the behavior of the cells in the absence of immunosuppression. Bisceglie did not recognize the impact of this approach for treatment of disease.

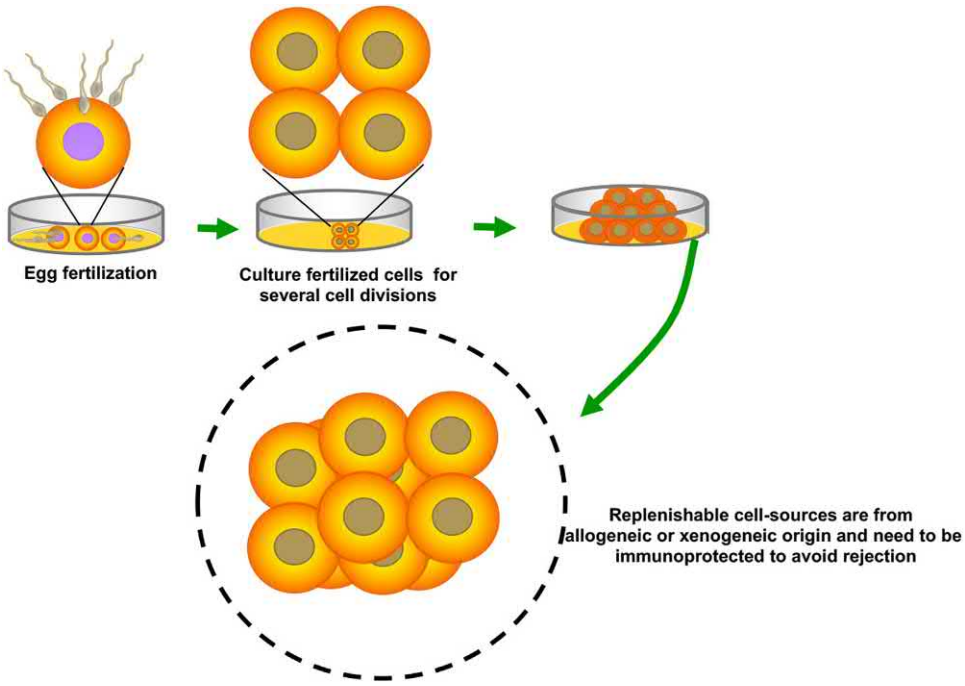

FIGURE 36.2 Replenishable insulin producing cell sources such as cells obtained from embryonic stem cell sources are either from allogeneic or even xenogeneic origin. To prevent graft rejection immunoisolation by encapsulation might be necessary.

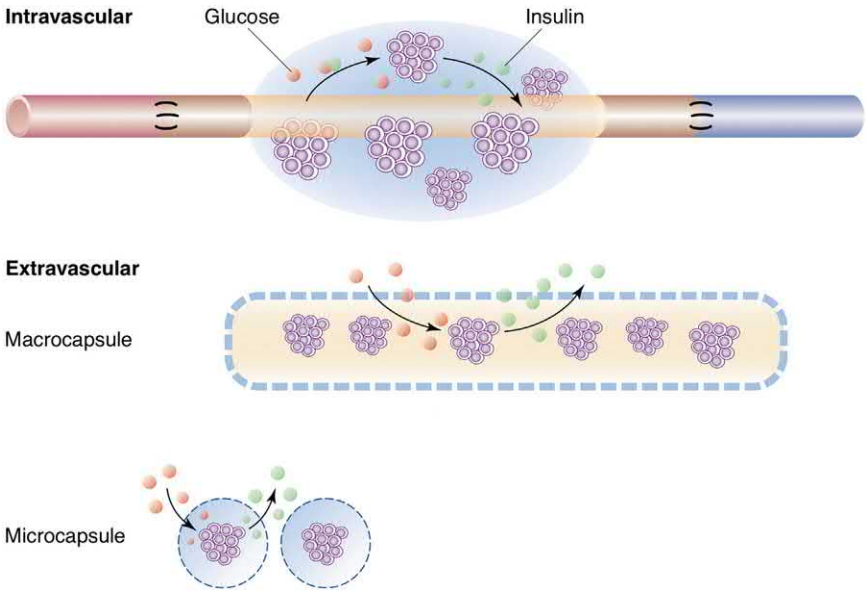

FIGURE 36.3 The bioartificial pancreas exist in three concepts. The intravascular macrocapsules, which allow fast exchange of glucose and insulin due to direct vascular access. The extravascular macrocapsules which can be implanted in the peritoneal cavity or under the skin and the extravascular microcapsules with an optimal volume to surface ratio that usually are implanted in the peritoneal cavity.

improved tremendously and some matured cells have near normal glucose-induced insulin release as early as 3 days after transplant [28]. There are however also other replenishable cell sources in development in which encapsulation might be helpful. For example, genome-editing technologies might also lead to new insulin-producing cell sources [29].

Macro- or microedevices

Currently there are three categories of devices under development for immunoprotection of insulin-producing cells: intravascular macrocapsules, extravascular macrocapsules, and extravascular microcapsules [30–32] (Fig. 36.3). All approaches have their pros and cons as will be discussed in the next section.

In intravascular devices, groups of islets are enveloped in relatively large diffusion chambers that protect the cells from the effector arm of the immune system. Intravascular devices are connected to the recipient's vascular system by anastomosis in most cases as arteriovenous shunt [33,34]. An advantage of intravascular devices is the fast exchange of nutrients, glucose, and insulin, which make a near

normal regulation of glucose metabolism possible. Because of these advantages the technology has received much scientific attention and was tested in both small and large mammals [35–39]. An issue with this approach is the chance of development of thrombosis, which might interfere with the patency of the device, and it might even happen that thrombosis occurs distant from the device [30,35]. High anticoagulation medication as well as the application of large bore devices was necessary to avoid patency issues and to guarantee flow-rate through the devices [40]. Especially the need for systemic and chronic anticoagulation therapy and also the relatively large size needed for the device were considered to be issues for clinical application. Development of these devices was stopped for almost two decades because of these issues but recently revisited. Recent developments in the field of lab-on-chip has led to the development of ultrafiltration membranes that provide better hydraulic permeability and an optimal permeability than could ever be achieved with the classical membranes that up to now were being applied in the intravascular bioartificial pancreas. With prototypes of these devices, clinically relevant numbers of islets could be kept viable and functional for prolonged periods of time [33]. The devices are proposed to be connected as arteriovenous shunt and should due to a difference in pressure between the artery and the vein result in an ultrafiltrate flowing through the device containing glucose and nutrients that flow through the small islet chamber inducing insulin release and adequate nutrition of the tissue. In vivo results are not available yet, but considering the enormous advances made in lab-on-a-chip technologies combined with the evolution of ultrafiltration techniques creates optimism about novel developments in the area of intravascular devices.

Extravascular devices in contrast to intravascular devices are not connected to the blood stream but implanted under the skin or in the peritoneal cavity without direct vascular access. For nutrition the cells in the device depend on free diffusion from the surroundings or on diffusion out of capillaries that grow in some concepts toward the surface of the device [30,41–43]. Extravascular devices are produced as macrocapsules or microcapsules. Macrocapsules can be produced from different types of polymers in the geometry of cylinders or disks or even as strings to which the islets are attached [43]. The macrocapsules usually contain groups of islets, which are enveloped in the device and subsequently implanted. For adequate nutrition, blood vessels should not be further away that 150–200 μm [44,45] to avoid formation of necrotic or necroptotic cells, which might leak immunogenic components into the surrounding. The technology is associated with not more than minor surgery and therefore considered to be a feasible approach for treatment of T1D. A major challenge, however, has been adequate oxygen supply toward the islets in the macrocapsules. Metabolic active islets require a high oxygen supply, which in most concepts of extravascular macrodevices is cumbersome due to an unfavorable surface-to-volume ratio. The competition for essential nutrients between islet cells in the device worsens this issue. As a consequence, with conventional macrodevices, the seeding density cannot exceed 5%–10% of the internal volume of the devices [46], which is a serious issue for clinical application as extremely large or several devices have to be implanted in human recipients to achieve efficacy.

This disadvantage of extravascular macrocapsules has partly been solved by inclusion of an external oxygen supply unit. The best studied approach with an external oxygen supplier is the so-called Beta-O2 device (Fig. 36.4). This device consists of two chambers. The inner chamber is the unit containing the insulin-producing cells, which are surrounded by the second chamber in which oxygen can be infused with a manually operated pump that funnels to the skin [47–50]. The inner chamber comprises a multilayer membrane providing immunoprotection. This membrane consists of a polytetrafluoroethylene mesh embedded in a high mannuronic acid alginate gel and a silicon rubber [45]. The multilayer membrane is freely accessible for essential nutrients such as oxygen and allows free exchange of glucose and insulin while providing immunoprotection [47]. In principle, this approach overcomes the issue of the low seeding density of the device and the need to use several or extremely large devices. Due to the versatile oxygen supply islet density can be increased and is much higher than in conventional extravascular macrocapsules. In the initial concept, 2400 IEQ/device were placed at a density of 1000 IEQ/cm^2 with refueling every 2 hours with O_2 [49]. This induced normoglycemia in diabetic rat

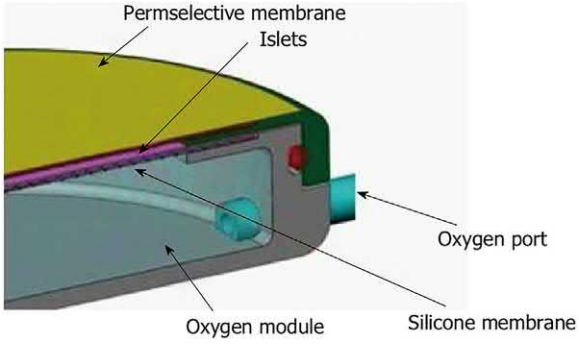

FIGURE 36.4 Schematic presentation of the Beta-O2 device. Within this concept islets are in larger groups encapsulated in semipermeable membranes. By inclusion of an oxygen chamber which can be refueled islets are provided with sufficient oxygen to generate adenosine triphosphate (ATP) and insulin. This allows higher seeding densities of islets than with conventional extravascular devices. This concept has successfully been tested in humans.

recipients for periods up to 240 days. In mini-pigs, which have a more complicated animal model with higher demands for both the device and the enveloped islets, efficacy was shown with a mass of 6730 ± 475 rat IEQ/kg [49]. Minimal responses against the devices were observed, and viable islets were retrieved after 3 months of implantation demonstrating the feasibility of the approach. During recent years, efficacy of the technology was shown in a human in an allogeneic setting and in nonhuman primates with xenogeneic porcine islets at a dose of only 20,000 islets/kg BW [51,52]. During reduction of exogeneous insulin supply, stable glycemic control was maintained for periods up to 190 days after which the devices were explanted [49]. After retrieval the devices showed optimal biocompatibility even in the human recipient and the device containing blood vessels in the immediate vicinity of the device [49]. It should be mentioned that the lower efficacy in larger animal models should not only be attributed to device issues but also to issues with islet-quality as finding suitable and high-quality islet sources for these types of experiments is still an issue and makes efficacy testing of the devices in larger mammals and humans complicated. Despite this, important advances have been made with macrocapsules, and supply of nutrients to the enveloped islets has created enthusiasm for human application of the technology when replenishable cell sources become available.

The other category of extravascular approaches is the microcapsules in which individual islets are enveloped in their own individual microcapsule. Microcapsules do suffer much less from oxygen supply or of lack of supply of other nutrients because of the more optimal surface-to-volume ratio. In our hands, if islets smaller than 150 μm are used, we observed no necrosis and optimal function for several months after implantation in the peritoneal cavity [53–56] (Fig. 36.5). Only larger islets (>250 μm) gradually develop central necrosis and cease to function after prolonged periods of implantation or culture [53–56], which might be prevented by reaggregating islets into smaller clusters [57]. Another advantage of microcapsules is that they can be made small enough to fit into organ structures with high vascularization degrees such as in the omental pouch although protrusion of cells is a challenge that needs to be solved in these approaches. Finally, microcapsules are because of their spherical small shape usually mechanically stable, and encapsulation can be done with nontoxic molecules and reagents [58] avoiding loss of valuable and rare donor tissue. This latter, that is, avoiding toxic reagents, is often an issue with macrocapsules and might lead to loss of tissue.

FIGURE 36.5 Neonatal pig islets after retrieval from a mice recipient at 8 weeks after implantation. If islets are smaller than 150 μm we do not observe any development of necrotic zone in islets.

The majority of studies addressing microencapsulation technologies use alginate as core material for the capsules. Alginates are available with different quantities of mannuronic acid (M) and guluronic acid (G), and suitability of different alginates depends on the system applied. To enhance permselectivity and mechanical stability, alginates are often coated with polyamide. Typically, for these systems a critical number of G–M polymers should be present as these G–M polymers are responsible for the binding of the polyamide structures on the surface of the capsules [59–61]. If cationic-based systems are used, other prerequisites apply to the alginate source. G–G blocks in alginate bind constitutive alginate molecules in a so-called egg-box model. These binding structures are responsible for the mechanical strength and stability of the system and therefore alginates with relatively high-G content are preferred. If, however, cations are applied with a higher affinity for alginate such as barium, it is not required to use high-G alginates as these cations bind both G–G and M–M. Typically, high-M alginates can only successfully be used with higher affinity cations such as barium [62]. This choice for the alginate type matching the system is an example of the complexity of the systems, which unfortunately in the field is not always acknowledged. This is one of the major reasons for the large variation in success rate of encapsulation systems when comparing different results of different labs. In many cases the type of polymer applied is not even documented or published illustrating a fundamental issue in this line of work [30,63].

Factors contributing to biocompatibility of encapsulation systems

In the past decade, many factors have been described, which contribute to prevention or are causing tissue responses against encapsulated cellular grafts. Biocompatibility is usually defined as “the ability of a biomaterial to perform with an appropriate host response

in a specific application." This definition was originally introduced for fully artificial organs, such as artificial prostheses [64]. Within the application of prostheses, a strong and immediate immune response is desired as it induces fibrosis of the prosthesis and integration into the surrounding tissue. The host response is desired as it allows integration of the prosthesis into the host tissue. For this reason, it is considered an "appropriate host response." For bioartificial organs such as the bioartificial pancreas that contain living cells, it is much more difficult to define "the appropriate host response." Fibrosis or integration into the surrounding tissue for extravascular devices is highly undesirable as it interferes with the nutrition of the enveloped tissue. Even a slight activation of the innate immune system may result in release of deleterious cytokines that might be harmful for encapsulated cells and should be avoided [65]. Preferably, the immune responses should be as minimal as possible to prevent massive cell-death, and to manage this response is far from easy [53,66–69].

In the past decade, much progress has been made in the field, and novel approaches to reduce or delete these responses have been published. These advances involve application of polymers/devices that lack proinflammatory residues, rational choices (combinations) of biocompatible polymers, insights into chemical conformations of surfaces to reduce host responses, and novel approaches for antibiofouling or immunomodulating biomaterials and application of polymers that form polymer brushes. These new developments will be discussed later.

Avoiding pathogen-associated molecular patterns in polymers

The role of pyrogenic contaminants and endotoxins in polymers in tissue responses against extravascular devices has been recognized for more than two decades [70,71] but is still influencing outcome of encapsulated islet grafts [41,68,72]. In recent years, we have developed a technology platform to identify possible immunogenic factors in polymers applied for cell-encapsulation and found that all currently commercially available polymers contain so-called pathogen-associated molecular patterns (PAMPs), including flagellin, lipopolysaccharide, peptidoglycan, lipoteichoic acid, and polyphenols [72]. The molecules are remnants of Gram-positive and -negative bacteria that come into the polymer preparation during processing, synthesis, or even during lyophilization procedures [72]. Some of these PAMPs classify as endotoxin but classical methods to quantify endotoxins such as the limulus amebocyte lysate assay does not react on many of these PAMPs and are consequently missed. Although regulations exist to which extend endotoxin levels are allowed in medical devices [63,67,73], we have the experience that these thresholds are far too high for bioartificial organs. Only devices with PAMP levels below the detection level did not provoke responses after implantation of the capsules in mice and rats.

PAMPs induce inflammatory responses in recipients either by diffusing out of the capsules or by being present at the capsule surface. This happens primarily by binding of the PAMP to pattern-recognition receptors (PRRs) on immune cells [74–76]. After the activation of PRRs on immune cells translocation of nuclear factor kappa-light-chain-enhancer of activated B cells occurs with inflammatory cytokine secretion as a consequence. This ultimately leads to adhesion of cells on the capsule surface and death of islet cells [77–80]. However, it should be emphasized that not only contaminants but also polymeric components can trigger these responses via PRRs. If wrong choices are being made for alginate sources uncross-linked mannuronic acid polymers can trigger immune activation via binding to Toll-like receptors (TLRs) [81]. These important novel insights have led to a broad acceptance that the field needs quality assessment systems for purity and immunogenicity of polymers applied in the devices [68,72].

Natural and synthetic polymers

Polymers applied in the bioartificial pancreas are both from natural or synthetic sources. As natural source it is mostly polysaccharides that are used in cell-encapsulation. The polysaccharides applied offer the advantage that they allow encapsulation of the cells under relatively mild conditions without affecting islet–cell viability [67]. Another reason why many favor polymers from natural sources is because hydrogels are formed that are as flexible as the surrounding tissue and still mechanically stable [82], while provoking minor or no host responses [20]. Synthetic polymers are also widely used and by some preferred because synthetic polymers hold the promise to allow reproducible production without batch-to-batch variations. Another reason why synthetic polymers are preferred is the ease by which they can be modified and tailor-made to improve biocompatibility or to generate other desired properties [83]. The type and suitability of different polymers applied in the field has extensively been reviewed [67,84–87] and will not be discussed in this chapter. Here we focus on the conformation of the molecules and how they can contribute to prevention of tissue response. Often this approach combines natural and synthetic polymers.

Multilayer capsule approaches

Many approaches where only one type of polymer was applied have significant shortcomings. For example, with

alginate, that is, the most commonly used polymer for cell-encapsulation, it was shown that when applied in low concentrations it was too porous to prevent ingress of immunoglobulin G [88,89]. Also, some systems with a monopolymeric approach were associated with low mechanical stability, higher surface roughness, and cell protrusions [58,90]. Because of these issues layer-by-layer systems were developed [91–93]. For alginate-based systems, mostly cationic polymers were applied to reduce permeability or enhance mechanical stability. This has evolved to development of layer-by-layer systems such as alginate-based systems coated with poly-L-lysine (PLL) [94], poly-L-ornithine [95,96], poly(ethylene glycol) (PEG) [97–99], chitosan [100–102], or agarose [103–107].

There is much misunderstanding and often lack of knowledge on the complexity of building an adequate polycationic membrane around alginate-based system. For example, for many years, PLL was considered to be a proinflammatory molecule leading to foreign body reactions [108–111]. New technologies in the field such as Fourier-transform infrared spectroscopy, X-ray photoelectron spectroscopy, and time-of-flight secondary ion mass spectrometry has revealed the essence of forcing PLL in certain complexes to avoid tissue responses [59,61,112–120]. First PLL-based system need alginates with an adequate number of G–M polymers as these are the molecules responsible for PLL binding. These alginates should first be gelified in a calcium solution and subsequently be treated with a buffer low in calcium but high in sodium. During this procedure the calcium at the surface is replaced by sodium which has a lower affinity for alginate than PLL. This facilitates binding of PLL in the next step that requires incubation in the absence of any divalent cations. If done properly this results in the formation of three layers: (1) random coil formations between alginate and PLL, (2) α-helicoidal structures between amide groups of PLL, and (3) antiparallel β-sheet structure between amide groups of PLL [59,113,121]. It is important that these formations are formed as these surfaces are not activating immune cells in the host. Any disturbance in the formation process can result in unbound cationic PLL structures at the capsule surface, which will lead to cell adhesion, immune activation, and inflammatory responses. We consider the insight in chemical structures responsible for induction of responses of uttermost importance as it leads to reproducible protocols to avoid these responses. For many systems it is still unknown which factors are critical, but we feel this should be a major focus for future research.

Antibiofouling approaches

Biofouling [122] is the process that every encapsulation device has to endure after implantation. It is the process that involves nonspecific adsorption of host proteins and subsequent immune cell and fibroblast adhesion. In most applications of encapsulation of insulin-producing cells, this process should be as minimal as possible as it affects viability of the enveloped cells. Ongoing research focuses on identifying molecules that can be grafted on the capsule surfaces that serve as so-called antibiofouling layer. Most strategies involve application of hydrophilic polymers that reduce protein adsorption [123]. One such molecule is PEG that in specific conformations is associated with low protein adsorption [124,125]. Efficacy depends on chain density, length, and conformation. Avoidance of protein adsorption of a PEG surface increases with higher polymerization degree. PEG has been successfully applied on alginate-based capsules as antibiofouling layer [91,126]. In this approach the PEG backbone was charged with amine groups (NH_3^+), in order to allow interaction with the negatively charged alginate. The antibiofouling PEG coating reduced fibrosis of the capsules and improved performance [126] but could not prevent graft failure. In another series of experiments the antibiofouling properties of PEG was applied by creating copolymers with PEG. PEG-block-poly(L-lysine hydrochloride) was used to bind to an alginate-based system. The PEG was protruding from the capsule surface, while the PLL was responsible for binding to the alginate. This approach significantly reduced proinflammatory responses [127]. The PEG of the diblock copolymer masked the proinflammatory PLL that was not bound in the above-described conformation and successfully formed an antibiofouling outer layer.

Formation of polymer brushes

Polymer brushes are polymeric chains that are densely packed and stretch on a surface [128]. Polymer brushes have been shown to reduce protein adsorption and cell adhesion in other fields of biomaterial research. It can be used to enhance biocompatibility but also to give surfaces of devices new functionalities [129]. Spasojevic et al. made polymeric brushes on capsule surfaces by applying novel diblock polymers such as diblock copolymers of PEG-block-poly(L-lysine hydrochloride) (PEG454-*b*-PLL100) [127,130] (Fig. 36.6). The copolymers are composed of a relatively small PLL tail that penetrates and binds onto the alginate network of capsule surfaces. The long PEG tail is at the outer surface after PLL binding onto the alginate network. At high densities the PEG is forced to stretch and form a brush. Only in certain combinations of molecular weight of PLL and PEG the brush can successfully be formed. This leads to a better biocompatibility and absence of cell adherence to the surface [127,130]. Other approaches to form brushes involves the use of a chitosan core on which polymer brushes of oligo

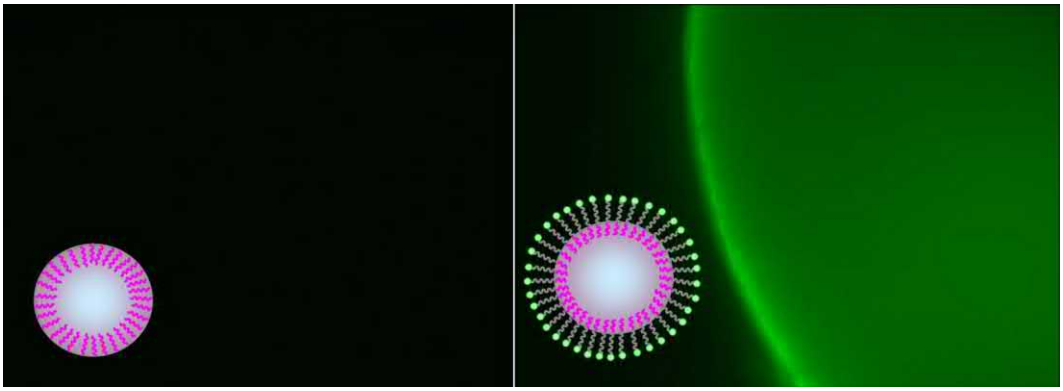

FIGURE 36.6 Confocal microscopy images after immunostaining of PEG blocks. Left are alginate-PLL100 capsules and right alginate-PLL100-PEG454-*b*-PLL50 capsules with a PEG454 brush. The illustration depicts the way the molecules are bound on the capsule surface. By using long molecular weight PEG a polymer brush is formed. Original magnification 10 ×. *PEG*, Poly(ethylene glycol); *PLL*, poly-L-lysine.

(ethylene glycol) methyl ether methacrylate and 2-hydroxyethyl methacrylate are formed by photopolymerization [131]. This brush reduced protein adhesion and eliminated platelet activation and leukocyte adhesion [131,132]. Although the formation of a brush is chemically challenging, the approach is reproducible and prevents influences on tissue responses of contamination of the polymers or inadequate formation of polycation layers [115,127]. For that reason, it holds many promises for future application in encapsulation devices.

Immunomodulatory materials

In the past decade the field of biomaterials has made important advances from which also the bioartificial pancreas does or will profit. Some biomaterials have been shown to modulate the immune system and by that can prevent undesired tissue responses [133–137]. One such an approach is the application of Staudinger ligation chemistry to link immunomodulatory molecules on, for example, PEG. This technology cross-links azide- and phosphine-labeled molecules and can be applied to couple biofunctional molecules with encapsulation polymers [138]. By this approach, immunomodulating thrombomodulin (TM) was coupled to PEG and immobilized on islet-cells through streptavidin–biotin interactions. TM inhibits proinflammatory cytokine production in macrophages and thereby reduces responses against coated mice pancreatic islets [139]. In another study, hemoglobin (Hb-C) was cross-linked with PEG to scavenge nitric oxide (NO) to reduce NO toxicity after immune activation [140,141]. Also, inclusion of silk has been reported to induce immunomodulation as after interaction with immune cells it decreased production of proinflammatory cytokines in vitro [142,143]. Silk in macrocapsules was able to change polarization of macrophages from a proinflammatory status to a regulatory M2 phenotype. This field is still in progress and more molecules applicable for the bioartificial pancreas will probably become available in the coming years.

Intracapsular environment and longevity of the encapsulated islet graft

It is broadly recognized that islet-derived factors released during the first phase after implantation negatively impacts the tissue responses with significant loss of islet cells as a consequence. This has led to new approaches in the past 5 years to preserve islet function and survival by inclusion of "survival" factors in the intracapsular milieu.

Tissue responses against an encapsulated graft already start with the mandatory implantation procedure. Although it is a minor surgery, it does lead to the release of proteins and cytokines to attract immune cells to delete possible pathogens that might be introduced during surgery. Under normal circumstances, this proinflammatory environment will soon be neutralized by IL-10 produced by other immune cells, but in case of implantation of a bioartificial pancreas, this process might get disturbed. Cells in the encapsulated graft not only release different types of molecules such as cytokines but also the so-called danger associated molecular patterns (DAMPs) that activate the local immune response [43,67,68,144–146]. These DAMPs are released by cells in the graft that are dying by necrosis or necroptosis [147,148]. Typical molecules that classify as DAMP are intracellular components such as DNA, RNA, and HMGB1 that bind to specific receptors on immune cells [149,150]. These receptors are called PRRs such as TLRs, nucleotide-binding oligomerization domain-like receptors, and C-type lectins [151–155]. The DNA, RNA, and HMGB1 that leak out of the capsules are very strong stimulators of PRRs and can enhance immunity and destruction of the graft [144]. These are all recent insights [144] that have demonstrated

that prevention of responses against bioartificial organs is not only determined by the biomaterials applied. Some modification of the capsules such as reducing permeability below 100 kDA can prevent leakage of some DAMPs, but despite this immune activation could not be completely prevented. Lower molecular weight DAMPs probably still diffuse out and induce immune activation [144]. Conceivable solutions to improve viability and reduce DAMP release are changing the intracapsular environment. It has been shown that inclusion of a necroptosis blocker called NEC-1 which is suppressing necrosis and necroptosis [144] reduces DAMP release and attenuates immune activation [144].

Another strategy to improve functional survival of cells in capsules is by inclusion of appropriate extracellular matrix (ECM) in the intracapsular environment. Islets are equipped with many integrins that interact with the ECM in their surroundings [156]. These interactions are essential for survival and serve many functions. ECM cannot only regulate via integrins the glucose-induced insulin release, proliferation, prevent or stimulate death signals but can also serve as a buffer for released cytokines and thereby conserve viability during immune attack [157–160]. A number of groups have applied laminin sequences such as tripeptide Arg-Gly-Asp (RGD) in their concept of encapsulation [161,162] and have reported long periods of graft survival in small as well as large animal models. We recently found that human pancreatic islets function longer and produce more insulin when they are encapsulated in a matrix containing collagen IV in combination with specific laminin sequences [57,163]. These combined observation illustrates that modifying the intracapsular environment is a feasible approach [156,163–165] to support functional survival of cells and prolong survival and diminish effects of immune responses against encapsulated grafts.

Concluding remarks and future considerations

In this chapter, we have reviewed some of the advances that have been made with the bioartificial pancreas. All three concepts, that is, intravascular devices, extravascular macrocapsules, and microcapsules, have undergone significant improvements in the past decade, and still all three might be developed in realistic approaches for treatment of T1D. An issue for many decades has been the undesired tissue responses against the devices that might lead to cellular overgrowth, which interferes with the functional survival of the cells. However, the introduction of novel polymeric approaches such as multilayer systems combining beneficial properties of several polymers but also the concept of polymer brushes and introduction of immunomodulating biomaterials has brought optimism that tissue responses can be reduced or even deleted. Unfortunately, there is still a large lab-to-lab variation in

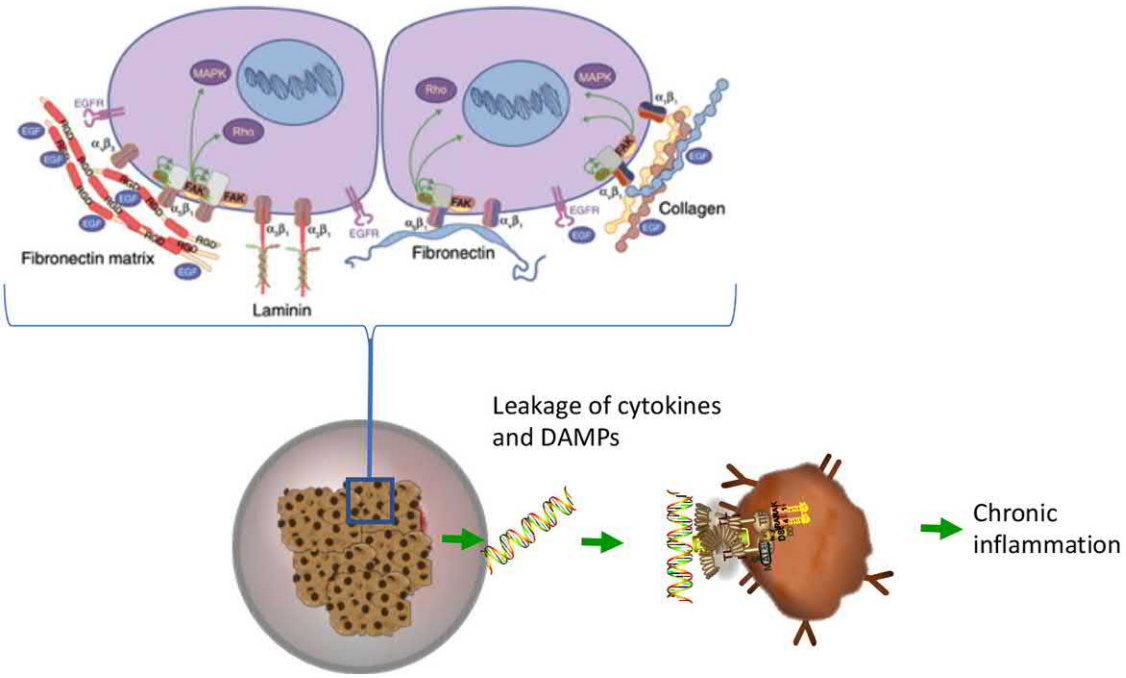

FIGURE 36.7 Cells in capsules need interactions with extracellular matrix molecules for cell homeostasis and survival. If interactions are not adequate cells may die and release danger associated molecular patterns (DAMPs) such as DNA, RNA, or HSP that bind to Toll-like receptors and other pattern-recognition receptors to induce proinflammatory responses. *DAMPs*, Danger associated molecular patterns.

outcome of encapsulated graft. This seems to be caused by lack of understanding of the need for application of specific molecules in specific systems and poor documentation of the procedures applied which make data comparison between labs cumbersome [30,63].

In recent years also, the intracapsular needs of islet-cells has gained attention. Current research efforts confirm that this is an essential, overlooked area in the transplantation field. Islets are equipped with integrins that need ECM contact. Many ECM molecules are destroyed during the enzymatic isolation of islet cells from the pancreas [166]. These molecules are required to allow survival of islets and also makes them more resistant to cytokine stress that is very common during the first week after implantation [164,167]. Significant improvements in functional survival of encapsulated islet cells are to be expected if specific combinations of ECM molecules are included in the intracapsular environment (Fig. 36.7). It has also recently been shown that many polymers used in biomedicine are poorly tolerated by islet-cells and may lead to dedifferentiation, lower glucose-induced insulin release profiles, and even cell-death [168,169]. This has received in the encapsulation field not more than minor attention but should be taken into account.

There are a few trends in the encapsulation field that need a critical debate. One of these is the current regulatory trend in the United States that some groups consider nonhuman primates the ultimate model for human application. As recently reviewed, this might be an essential mistake [72]. Nonhuman primates have innate and adaptive immune responses that are very different from human responses against cellular grafts. Especially, the innate immune pathways are different in nonhuman primates [72]. Much more information can be obtained from rodent studies with a humanized immune system and from small clinical trials.

We have reviewed in this chapter not only the progress but also remaining challenges for human application. These challenges are not all related to the capsules or biomaterials. A major challenge is to find adequate sources for insulin-producing cells. Many groups, including ours, wish to start human trials but adequate insulin-producing cell sources are lacking. High-quality human islets are difficult to obtain in sufficient amounts to do human trials. Pig islets and insulin-producing cells obtained from progenitor cells and stem cells still have functional limitations. This also limits the preclinical and clinical testing of the devices. Several concepts have a fair chance on success but adequate insulin-producing cell sources are required to come to testing and wide-scale application of the bioartificial pancreas.

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Chapter 37

Thymus and parathyroid organogenesis

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Structure and morphology of the thymus

The mature thymus is a highly dynamic cellular environment. Developing T lymphocytes (thymocytes) make up over 95% of its cellularity, with the remaining few percent comprising the complex stromal compartment, which is composed of mesenchymal cells, bone marrow (BM) derived macrophages and dendritic cells (DCs), vasculature, and the uniquely specialized thymic epithelium (TE) [1]. The organ is encapsulated and lobulated and contains three principal histologically defined regions, the cortex, the medulla, and the subcapsule (Fig. 37.1). The capsule and trabeculae consist of a thick layer of connective tissue and are separated from the cortex by the subcapsule, a thin layer of simple epithelium [1]. The cortex and medulla each contain open networks of epithelial cells that are densely packed with thymocytes [1–3], and each of the cortical and medullary regions contains several different distinct epithelial subtypes (as discussed later). These cortical and medullary thymic epithelial cells (cTEC and mTEC, respectively) are key elements of the thymic stroma, providing many of the organ's specialist functions. The outer cortex also contains fibroblasts, with further complexity of the thymic fibroblast compartment beginning to emerge. The organ as a whole is heavily vascularized. BM-derived stromal cells also exist and are found in both compartments, macrophages being distributed throughout the organ, while thymic DCs—which are required for imposition of tolerance on the emerging T cell repertoire—are found predominantly at the corticomedullary junction (CMJ) and in the medulla [1].

Thymus structure is intimately linked to its function, which is to support development of a functional, self-tolerant T cell repertoire. This encompasses the linked processes of T cell differentiation and T cell repertoire selection, which together ensure that the peripheral T cell repertoire is predominantly populated by T cells that respond to antigens in the context of self-major histocompatibility (MHC) antigens, but which are not activated by binding self-peptides.

T cell development has been extensively reviewed elsewhere [4–11] and is not discussed in detail herein. In brief, thymocyte progenitors enter the postnatal thymus through blood vessels at the CMJ, and subsequent T cell development is then regulated such that thymocytes at different stages of development are found in different intrathymic locations. The earliest thymocyte progenitors do not express the CD4 or CD8 coreceptors [part of the T cell receptor (TCR) complex] and are referred to as double negative (DN) cells. DN thymocyte progenitors undergo a highly ordered series of maturation stages in the thymic cortex and upregulate expression of CD4 and CD8 to become the major double positive (DP) thymocyte subset. The cortex itself can be subdivided into four regions based on the localization of thymocyte populations at different stages of development. Thus zone 1 contains the colonizing population of thymocyte progenitor cells; these early thymocytes undergo proliferative expansion in zone 2; T cell lineage commitment is completed in zone 3; and in zone 4, thymocytes differentiate to the DP stage of development, characterized by expression of both CD4 and CD8 coreceptors [12,13] (see Fig. 37.1). Only DP thymocytes expressing TCRs that mediate low affinity interactions with self-peptide/MHC complexes, presented on cTEC, are positively selected. These positively selected thymocytes survive and mature into $CD4^+$ or $CD8^+$ single positive (SP) cells. Thymocytes that have been positively selected then migrate into the medulla [14,15]. The remaining DP thymocytes, which fail

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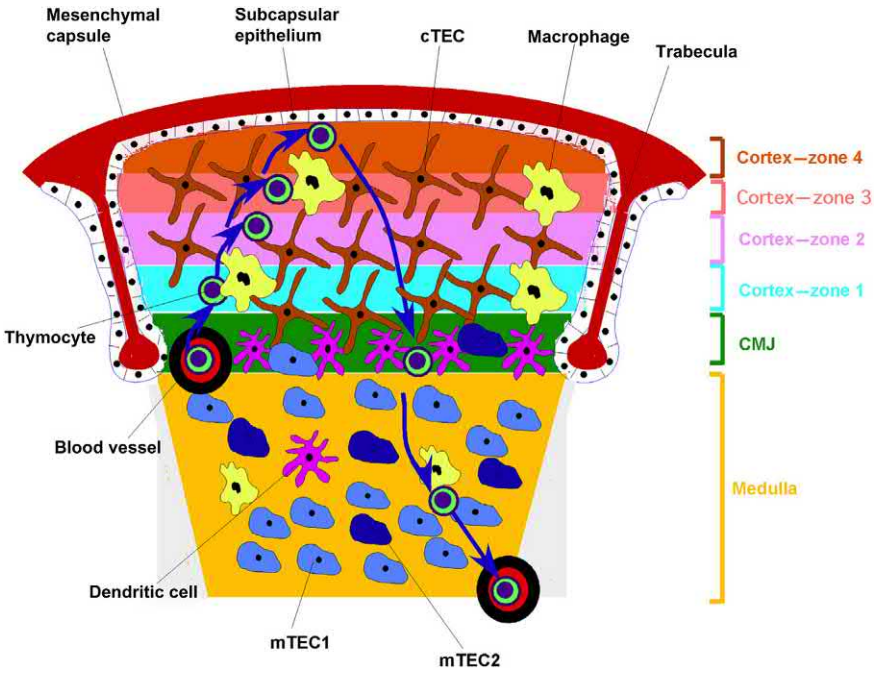

FIGURE 37.1 Histology of the postnatal thymus.
The postnatal thymus is surrounded by a capsule consisting of mesenchymal cells and connective tissue, which penetrates into the thymus at regular intervals to form trabeculae. Underlying the capsule and trabeculae is the subcapsular epithelium consisting of a layer of simple epithelium, which overlies the outer cortex. The cortex is populated with cTEC, macrophages, and developing thymocytes at the DN and DP stages of development. Thymocytes enter the thymus at the CMJ via the vasculature and migrate through the cortex to the subcapsule as they differentiate. The cortex can be divided into four zones based on the differentiation status of thymocytes that reside within it. Thus zone 1 contains the most immature DN1 thymocytes and zone 4 contains thymocytes undergoing the DN4-DP transition. DP thymocytes are then screened for propensity to recognize self-MHC, a process termed "positive selection," and those selected to mature into $CD4^+$ or $CD8^+$ SP cells migrate into the medulla, where they undergo the final stages of maturation before being exported to the periphery. Central tolerance is established by deletion of self-reactive thymocytes in a process termed "negative selection" and occurs principally at the CMJ and in the medulla: negative selection is mediated both by thymic dendritic cells and medullary TECs. Medullary TECs are also required for the generation of $CD4^+CD25^+$ T regulatory (Treg) cells and natural killer T cells, both of which actively repress self-reactive T cells. *CMJ*, Cortico-medullary junction; *cTEC*, cortical thymic epithelial cells; *DN*, double negative; *DP*, double positive; *MHC*, major histocompatibility; *SP*, single positive; *TEC*, thymic epithelial cell.

positive selection, die by apoptosis [16]. Intrathymic migration of thymocytes is at least partly a regulated process. Both the outward migration of thymocytes from the CMJ to the outer cortex [17] and the migration of positively selected cells from the cortex into the medulla [5,15,18–21] are controlled by chemokines.

In the medulla, a wide array of self-antigen/MHC complexes presented by mTECs and/or DCs induce apoptosis of those SP thymocytes that express TCRs with high affinity for self-antigens [22–24]. This process, termed "negative selection," is essential to reduce the frequency of autoreactive T cells in the peripheral T cell repertoire. SP thymocytes proliferate and undergo the final stages of T cell maturation in the medulla before emigrating from the thymus to enter into the peripheral immune system. Some high affinity self-reactive thymocytes escape negative selection and instead differentiate into T regulatory (Treg) cells, which suppress autoreactive T cells in the periphery and are therefore an important component of self-tolerance [25–27].

Thymic epithelial cells

Complexity of the thymic epithelium compartment

The TE is highly heterogeneous in terms of immunophenotype and function, and recapitulation of this heterogeneity is likely to prove essential for the development of a truly functional engineered thymus. Ultrastructural and immunohistochemical analyses initially revealed six different subtypes of TEC [28–31], as well as large complexes of TECs and developing thymocytes [31] termed "thymic nurse cells" [32,33]. In addition, some epithelia identified in small isolated clusters at the CMJ were classified as undifferentiated [31] and proposed as precursors of differentiated TEC [34]. Different TE subpopulations can be defined by differential expression of cytokeratins (K) and other markers including MHC Class II, CD40, and CD80. All TEC express MHC Class I, while MHC Class II, CD40, and CD80 expression is variable [35–38]. In both the cortex and the medulla, high levels

of CD40 and surface MHC Class II are thought to identify the most mature TECs [39–41].

Two cTEC populations have been identified based on cytokeratin expression—a predominant $K5^{-}K14^{-}K8^{+}K18^{+}$ subset and a minor subset consisting of $K5^{+}K14^{-}K8^{+}K18^{+}$ cells that is found at the CMJ and scattered throughout the outer cortex [42]. Most if not all cTECs also express the determinants recognized by mAbs Ly-51 [43], which recognizes the homodimeric cell surface glycoprotein BP-1, and CDR1 [44], the target of which is not yet biochemically defined. cTEC additionally express the endocytic receptor CD205 [45–47], and the TEC-specific catalytic proteasome subunit, β5t [48–50].

Most medullary (m) mTEC are $K5^{+}K14^{+}K8^{-}K18^{-}$ [42] and also bind mAb MTS10 [30], while a minor $K5^{-}K14^{-}K8^{+}K18^{+}MTS10^{-}$ mTEC subset also exists [42]. mTECs also express high levels of epithelial cell adhesion molecule (EPCAM) as determined by immunohistochemistry [51] (all TEC are $EPCAM^{+}$ by flow cytometry) and bind the lectin *Ulex europaeus agglutinin-1* (UEA-1) [52], with a subset of $K14^{-}$ mTECs binding UEA-1 at high levels. mTECs can be further subdivided based on CD80 expression, the level of which correlates with degree of functional maturation as for MHC Class II [40,53]. The lineage relationships between these different TEC subsets remain incompletely defined (see later). Furthermore, while these analyses give an indication of the complexity of the TE, they underestimate the number of TEC subtypes present in the adult thymus based on recent transcriptome analysis [54–56].

Functional diversity

The functional dichotomy between the cortical and medullary compartments of the mature thymus (see the "Structure and morphology of the thymus" section) is reflected in functional differences between cTEC and mTEC. cTEC express Delta-like 4 (DLL4), the Notch ligand required throughout the lifespan for commitment of thymocyte progenitors to the T cell lineage;[57–59] membrane-bound Kit ligand (mKITL), required by DN2 thymocytes (note that mKITL presented by cortical vascular endothelial cells is also required at the DN1 stage); [60] and the chemokine CXCL12 [also known as stromal cell-derived factor 1 alpha (SDF1α)], a regulator of the β-selection checkpoint that controls progression from the DN to the DP stage of thymocyte development [61–63]. In addition, cTEC express ligands required for positive selection [64]. Notably, β5t expression in cTEC is required for generating an optimally selected $CD8^{+}$ T cell repertoire. The β5t-containing "thymoproteasome" generates a specific set of self-peptides for presentation by MHC Class I, and in the absence of β5t, a depleted, functionally deficient $CD8^{+}$ T cell repertoire is selected [49,65–72]. Similarly, expression of Cathepsin L (Cathepsins L and V in human) in cTEC is required for optimal positive selection of the $CD4^{+}$ T cell repertoire [73–76].

mTECs play an essential role in the development of central tolerance, by mediating both negative selection of the T cell repertoire and development of Tregs. In addition, they regulate the migration of positively selected thymocytes into the medulla via the expression of CCL19 and 21 [15,18,19,77], with expression of CCR4 ligands by medullary DC also contributing to regulation of this process [78,79]. mTECs regulate central tolerance through expression of a broad repertoire of proteins, termed tissue-restricted antigens (TRAs), that are otherwise tissue or developmentally restricted [80–82]. Expression of a subset of these genes is regulated by the *Autoimmune Regulator, Aire*, which, within the thymus, is specifically expressed in $CD80^{hi}$ mTEC [83–89], with individual TRAs being expressed stochastically within the total $CD80^{hi}$ mTEC population [90]. mTEC-expressed TRAs are presented to medullary thymocytes by both mTECs and DCs, the latter via a cross-presentation mechanism [24,91,92]. In the absence of AIRE, mice and humans develop the broad-spectrum autoimmune syndrome, APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) [86,87]. AIRE-independent expression of TRAs by mTECs may be regulated by the transcription factor Fez Family Zinc-finger 2 (FEZF2) which, in contrast to AIRE, is expressed in both $CD80^{lo}$ and $CD80^{hi}$ mTECs [93].

The medulla is also distinguished by corpuscular bodies of epithelial cells known as Hassall's corpuscles. These are particularly prevalent in human thymus and have been shown to express thymic stromal lymphopoietin, a signaling molecule that instructs closely associated DCs in the medulla to induce Treg cells [25,26]. In this regard, mTEC also regulate the accumulation and positioning of DCs in the medulla via the secretion of the chemokine XCL1 [94]. Further complexity in the mTEC compartment has emerged with the identification of "post-AIRE mTEC" [95,96], and through single-cell RNAseq analyses which reveal the presence of at least four major mTEC subtypes, including a subpopulation with Tuft cell–like identity [55,56].

In vitro T cell differentiation

The high level of phenotypic and functional heterogeneity demonstrated above presents a significant challenge for attempts to fully recapitulate thymus function in vitro and is also highly pertinent to cell replacement or regenerative strategies for enhancing thymus activity in vivo. Currently, it is not possible to reconstitute full thymus function in vitro or upon transplantation except in cultures

based on ex vivo thymus tissue. However, in vitro systems that fully or partly support T cell differentiation are widely used as a tool for investigating regulation of this process, and recently, the use of in vitro culture of hematopoietic progenitors to enhance lymphohematopoietic reconstitution following BM transplantation has been investigated. Current approaches to in vitro T cell generation are summarized later.

Fetal thymic organ culture (FTOC) utilizes ex vivo thymic lobes, usually derived from E15.5 to E16.5 mouse embryos or second-trimester human fetuses, to support the differentiation of T cell progenitors from endogenous or exogenous sources [97–101]. The technique of reaggregation fetal thymic organ culture (RFTOC), in which defined TEC subpopulations are obtained by cell purification techniques, reaggregated with fibroblasts, and defined lymphocyte populations, and then cultured further in vitro, was developed as an extension of FTOC and has proved invaluable for assessing the role of individual stromal components during specific stages of T cell maturation [64,102]. This approach has also been combined with grafting, typically under the kidney capsule, since grafted RFTOC typically exhibit better cortical and medullary patterning than in vitro cultured RFTOC and has been adapted for testing the potency of different fetal and adult TEC subpopulations from mouse, rat, and human [103–110]. Improved reproducibility of the RFTOC technique was demonstrated using an approach for cellular reaggregation termed Compaction Aggregation (CoROC), which also permits the juxtaposition of the different cell types included in the cellular reaggregate to be controlled [111].

A related approach demonstrated the generation of an in vitro "thymic organoid" by seeding a tantalum-coated carbon matrix with ex vivo murine thymic stromal cells [112]. When these structures were cocultured with human $CD34^+$ hematopoietic progenitors, efficient generation of mature $CD4^+$ and $CD8^+$ SP T cells was observed after 14 days. The T cells generated in this system were functional, as demonstrated by their proliferative response to mitogenic stimuli, and demonstrated TCR repertoire diversity comparable to that of peripheral blood T cells [112]. These findings established that the utilization of three-dimensional matrices in conjunction with thymic stromal cells can provide an efficient and reproducible method of in vitro T cell generation. However, this approach relies on seeding with ex vivo thymus tissue and therefore is not highly scalable.

The demonstration that transfection of the BM stromal cell line OP-9 with the Notch ligand Delta-like 1 (DL1) conferred the capacity to support T cell differentiation from a variety of hematopoietic progenitors in monolayer culture represented a major breakthrough [113,114]. Using this system, DP thymocytes can be generated efficiently from mouse fetal liver, adult BM, or embryonic stem (ES) cell-derived hematopoietic progenitors; $CD8^+$ SP T cells are also produced inefficiently although $CD4^+$ SP T cells are largely absent [113,114]. This system was also shown to support T cell development from human cord blood– and human BM–derived $CD34^+$ cells [115–118]. OP-9 cells stably transfected with Delta-like 4 (DLL4), the nonredundant Notch ligand required for T cell lineage commitment in vivo [57,58], also efficiently support the generation of DP cells [119,102]. In the OP-9 system, lower levels of DLL4 than DL1 are required for T lineage commitment [119].

Neither the OP-9-DL1 nor the OP-9-DLL4 system can be used to generate mature T cells for transplantation into patients [120] since OP-9 cells lack the cTEC and mTEC-specific gene-expression programs required to direct physiological positive and negative selection of the T cell repertoire. However, these systems have been widely used to dissect cellular and molecular regulation of T cell differentiation in mouse and human (see, e.g., Refs. [13,121]). In addition, they have been used to produce expanded pools of hematopoietic progenitor cells (HPCs) for transplantation [122–126]. In particular, adoptive transfer of OP-9-DL1-produced $CD4^-CD8^-$ DN thymocytes in a mouse model of hematopoietic stem cell (HSC) transplantation resulted in improved T cell reconstitution [122]. Similarly, the transplantation of human $CD34^+CD7^{hi}$ cells produced by in vitro culture of umbilical cord blood or adult HSC resulted in the efficient engraftment of the thymus of immunodeficient mice [123,125]. Furthermore, in preliminary clinical trial data, transplantation of OP-9-DL1-cultured HPC, together with a single unit of cord blood, resulted in more rapid recovery of neutrophil numbers following a myeloablative preparative regimen than the currently standard procedure of transplantation of a double unit of cord blood [124].

The successful application of OP9-DLL1-derived $CD4^-CD8^-$ DN thymocytes in T cell reconstitution has led to the development of stromal cell free culture systems. Culture systems based on immobilized DLL1 [127] and DLL4 [128] have shown potential to expand lymphoid precursor cells in vitro and in vivo. Cord blood $CD34^+$ HSCs cultured on immobilized DLL1 led to the expansion of $CD34^+CD7^+$ pro-T cells, capable of thymic repopulation in immunodeficient mice [127]. Expansion of $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) on immobilized layers of DLL4 and VCAM-1, a potent enhancer of Notch activation, leads to the maturation of $CD7^+$ pro-T cells, capable of reconstituting the periphery of immunodeficient mice [129]. HSPCs cultured on immobilized DLL4 and VCAM-1 for 14 days prior to engraftment into immunodeficient mice were shown to have homed to the thymus by 4 weeks, and at 10–12 weeks postengraftment human $CD3^+CD8^+$ T cells were isolated from the peripheral blood, indicating that pro-T

cells developed on immobilized Notch ligands are capable of reconstituting the peripheral immune system. In addition to the OP9-based systems, a serum-free "artificial thymic organoid" (ATO) in vitro T cell differentiation system based on the mouse BM cell line MS5 transduced with human DLL1, has recently been described [130]. Three-dimensional organoids based on this cell line efficiently support human T cell development to the DP and $CD8^+$ SP stages, with some mature $CD4^+$ SP also developing probably as a result of selection on DCs within the ATO [130]. This system, in combination with an embryonic mesodermal induction step, has also recently been shown to support differentiation of human T cells from human pluripotent stem cells [131].

Thus in vitro approaches based on generating and/or expanding HPC appear poised for translation. Progress toward the goals of generating transplantable T cell repertoires in vitro, that have undergone physiological positive and negative selection, however, is likely to require a combination of stem cell–based approaches coupled with tissue engineering to pattern the resulting organoid.

Proof-of-principle studies have also demonstrated the feasibility of restoring adaptive immunity in patients by thymus transplantation [132–134]. This approach currently relies on the transplantation of human neonatal thymus tissue, and, in addition, a high proportion of recipients develop autoimmune complications [135]. These issues collectively limit the widespread use of the current thymic transplantation approach.

In this regard the use of decellularized thymic extracellular matrix (ECM) for developing transplantable tissue-engineered thymic organoids has recently been investigated. The earliest report described the use of a detergent-perfusion system to decellularize 3–4-week postnatal thymi, which were then seeded with an enriched preparation of ex vivo thymic stroma, plus HSCs [136]. However, the interpretation of the results of this study was confounded by limitations associated with experimental design and controls. The enriched thymic stromal preparation still contained a substantial (18%) population of SP thymocytes that possess the potential to populate the periphery of the haplotype-matched nude donor without undergoing a further, thymus-dependent differentiation process and may therefore be responsible for the functional effects the authors ascribed to the ECM-scaffold [136].

Subsequently, the zwitterionic surfactant 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) was used to prepare an ECM gel from postnatal thymi obtained from mice at an age of 7 weeks. These CHAPSO-derived scaffolds were seeded with E14.5 $CD45^-EPCAM^+$ TEC and $CD45^-EpCAM^-$ thymic mesenchyme and were able to mediate thymopoiesis from recruited hematopoietic precursors. Compared to control RFTOC-kidney grafts, the CHAPSO-scaffolds significantly improved TEC and thymic mesenchyme proliferation and the contribution of T cells to the peripheral immune system. However, this study did not directly investigate T cell function or central tolerance induction [137].

These pioneering studies together suggest that the integration of stem cell technologies, to provide an alternative source of thymic cells, with tissue engineering may eventually allow thymus transplantation to be more widely adopted: in addition to the challenge of generating a thymus by tissue engineering, the cause of autoimmunity in current thymus transplantation protocols must be understood and addressed. In the following sections, we summarize current knowledge of molecular and cellular control of thymus development and maintenance as related to these aims. We focus primarily on the TEC compartment, based on the critical function of these cells in the thymus. We also discuss the current understanding of parathyroid development, since parathyroid replacement is also a clinically important goal.

Thymus organogenesis

Cellular regulation of early thymus organogenesis

The thymus arises from a common primordium with the parathyroid gland. This common primordium develops from the third pharyngeal pouch (3PP), one of a series of bilateral outpocketings of pharyngeal endoderm. In the mouse, outgrowth of the 3PP occurs from approximately E9.0 [138]. At this stage the epithelium of the 3PP consists of a single layer of columnar epithelium surrounded by a condensing population of neural crest cells (NCCs) that will eventually form the capsule and thymic pericyte populations [139–142]. Overt thymus organogenesis is evident from between E10.5 and E11.0, at which stage the epithelium begins to proliferate, assuming a stratified organization [143]. At E12.5 the primordia separate from the pharynx and begin to resolve into discrete thymus and parathyroid organs. The thymus primordium subsequently migrates to its final anatomical location at the midline, following the path of the carotid artery and vagus nerve, while the parathyroid primordium associates with the lateral margins of the thyroid [144,145]. In the case of the thymus, this migration is active and is regulated by NCC through EphB-ephrin-B2 interactions [146–148]. Within the common primordium the prospective thymus is located in the ventral domain of the 3PP, and the prospective parathyroid in the dorsal aspect. Patterning of these prospective organ domains occurs early in organogenesis, as the parathyroid domain is delineated by the transcription factor GCM2 as early as E9.5 (see the "Specification of the thymus and parathyroid" section).

The mesenchymal capsule surrounding the thymus primordium is derived from the migratory neural crest, a transient population formed between the neural tube and the surface ectoderm. In the mouse, NCCs migrate into the pharyngeal region from E9.0. Elegant chick-quail chimera studies provided the first evidence that NCCs are the source of mesenchymal cells in the thymus [140], which was confirmed in the mouse by heritable genetic labeling in vivo [139,141,142].

Colonization of the mouse thymus with HPCs occurs between E11.25 and E11.5 [149–151]. As vascularization has not occurred by this stage, the first colonizing cells migrate through the perithymic mesenchyme into the TE [152]. These cells have been reported to exhibit comparatively low T cell progenitor activity, while a second colonizing wave that arrives between E12 and E14 appears to display much higher levels of T cell potential upon in vivo transfer [153].

Following the formation of the thymic primordium and the commitment of the epithelial cells to the TEC lineage, the thymus undergoes a period of expansion involving both the proliferation of stromal cells and an increase in thymocyte numbers. In vitro experiments have demonstrated that both FGF7 and FGF10, which are expressed by the perithymic mesenchyme [154], can stimulate the proliferation of fetal TECs [155,156]. Furthermore, thymi in mice lacking *Fgfr2IIIb*, the receptor for FGF7 and FGF10, are severely hypoplastic although able to support T cell differentiation, and $Fgf10^{-/-}$ mutants also exhibit hypoplastic thymi [154]. Similarly, the expression of a soluble dominant negative FGFR2IIIb fusion protein by thymocytes resulted in reduced thymic size and cellularity, although thymocyte development was unperturbed [157].

Concomitant with this proliferation TEC differentiation commences with the initial stages of differentiation into cortical and medullary cell types evident by E12.5 [41,105]. The development of the two compartments then proceeds in a lymphocyte independent manner until E15.5 [158,159]. The expression of MHC Class II and MHC Class I on the surface of TEC is first detected at E12.5, and ~E16, respectively [36,41,160,161] and is followed by the appearance of $CD4^+$ and $CD8^+$ SP thymocytes at E15.5 and E17.5 [36,160]. Although a functional thymus is present in neonates, the full organization of the stroma is not achieved until 2–3 weeks postnatally in the mouse.

We note that the precise timings of developmental events varies between mouse strains, and the Blackburn lab has observed that the thymic primordium in C57BL/6 embryos is consistently developmentally retarded by at least 12 hours compared to CBAxC57BL/6 F1 embryos at E12.5. Hence, the timings reported for particular events in thymus organogenesis may vary slightly due to the variations in genetic background.

Origin of thymic epithelial cells

The precise embryonic origins of the TE were controversial for many years [104,150,162–167]. However, evidence obtained using chick-quail chimeras showed that the epithelial compartment of the thymic stroma was derived solely from the pharyngeal endoderm [164]. Subsequently, definitive evidence for a single endodermal origin in mice was provided through histological, fate, and potency analysis of the pharyngeal region [138]. In particular, transplantation of 3PP pharyngeal endoderm isolated from E8.5 to E9.0 embryos (i.e., prior to initiation of overt thymus organogenesis) established that grafting of this endodermal region was sufficient to direct complete thymus organogenesis, showing that pharyngeal endoderm alone is sufficient for the generation of both cortical and medullary thymic epithelial compartments [138].

Thymic epithelial progenitor cells

The phenotype of thymic epithelial progenitor cell (TEPC) has been of considerable interest and remains an area of ongoing investigation. Analysis of a subset of human thymic epithelial tumors initially suggested the existence of a common TEPC; these tumors contained cells that could generate both cortical and medullary subpopulations, thus suggesting that the tumorigenic targets were epithelial progenitor/stem cells [168]. In addition, ontogenic studies suggested that the early thymus primordium in both mouse and human might be characterized by the coexpression of markers that later segregated to either the cortical or medullary epithelium [169].

The first genetic indication of a TEPC phenotype was provided by a study addressing the nature of the defect in *nude* mice [170] that fails to develop a functional thymus due to a single base deletion in the transcription factor FOXN1 [171,172]. Analysis of allophenic *nude*-wild-type aggregation chimeras demonstrated that cells homozygous for the *nude* mutation were unable to contribute to the major TE subsets, establishing that the *nude* gene product (FOXN1) is required cell-autonomously for the development and/or maintenance of all mature TEC [170]. However, a few *nude*-derived cells were present in the thymi of adult chimeras, and phenotypic analysis indicated that these cells expressed determinants reactive to mAbs MTS20 [30] and MTS24 (both subsequently shown to bind a determinant on the PLET1 protein [173]) but did not express markers associated with mature TEC including MHC Class II. These findings suggested that in the absence of *Foxn1*, TE lineage cells underwent maturational arrest and persisted as $MTS20^+24^+$ progenitors [170]. This hypothesis was confirmed by clonal reactivation of a conditional null allele of *Foxn1* in the neonatal thymus, which resulted in the generation of functional

thymus tissue containing organized cortical and medullary regions [174]—demonstrating unequivocally that in the absence of FOXN1, a common TEPC persists in the thymic rudiment. A similar approach using conditional reversion of a severely hypomorphic allele of *Foxn1* subsequently demonstrated that in the absence of functional levels of FOXN1 expression, this common TEPC is stable for at least 6 months in vivo [175].

Further data regarding the phenotype of TE progenitors came from the analysis of mice with a secondary block in thymus development resulting from a primary T cell differentiation defect. The thymi of postnatal *CD3e26tg* mice, in which thymocyte development is blocked at the $CD44^{+}CD25^{-}$ TN1 stage [176,177], principally contain epithelial cells that coexpress K5 and K8 [42]—which, in the normal postnatal thymus, are predominantly restricted to the medulla and cortex, respectively, and are coexpressed by only a small population of TEC at the CMJ. Klug et al. demonstrated that transplantation of *CD3e26tg* thymi into $Rag1^{-/-}$ mice, which sustain a later block in T cell differentiation, resulted in the development of $K5^{-}K8^{+}$ cells, suggesting that the $K5^{+}K8^{+}$ cells are progenitors of cTEC [42].

The phenotypic and functional properties of $MTS20^{+}24^{+}$ cells within the fetal mouse thymus were addressed directly in several studies. Analysis of the functional capacity of isolated $MTS20^{+}24^{+}$ cells and $MTS20^{-}24^{-}$ cells via ectopic transplantation demonstrated that $MTS20^{+}24^{+}$ TEC were sufficient to direct the establishment of a functional thymus containing both cortical and medullary TEC populations [105,106]. These studies clearly identified fetal $MTS20^{+}24^{+}$ TEC as TEPC but lacked clonal evidence for the existence of a common TEPC. Expression profiling subsequently identified the target of MTS20 and MTS24 as the orphan cell surface protein PLET1 [173].

Evidence for a common TEPC has been presented in two independent studies. A short-term retrospective clonal lineage analysis demonstrated the presence of both a common TEPC able to generate both cTEC and mTEC, and cTEC-restricted TEPC, in the neonatal thymus [174]. In an elegant extension of these experiments, clonal reversion of a *Foxn1* null allele resulted in the generation of small regions of thymus tissue that contained both cortical and medullary TEC [174], providing conclusive evidence for the existence of a common progenitor in $Foxn1^{-/-}$ mice, as discussed above. In a complementary study the existence of a common TEPC in the E12.5 thymus was demonstrated by the transplantation of single E12.5 $PLET1^{+}$ TEC [108], establishing that the $PLET1^{+}$ TEC population originally identified as TEPC indeed contains a common TEPC.

The view that PLET1 is a marker for TEPC was challenged in a report demonstrating that at E14.5, both the $PLET1^{+}$ and $PLET1^{-}$ TEC compartments can form a functional thymus upon transplantation [178], in contrast to initial conclusions [106]. However, a key difference between these studies is that Gill et al. transplanted limiting numbers of $PLET1^{+}$ TEC [106], whereas in the later study, cell number was not limiting [178]. Furthermore, Rossi et al. did not provide phenotypic analysis of the input populations and therefore could not determine precursor:progeny relationships—a caveat of particular importance since the PLET1-negative TEC population at E14.5 and subsequent developmental stages is highly heterogeneous [41]. Rossi's findings could thus be consistent with the existence of intermediate progenitor populations restricted to cortical and medullary TEC fates downstream of $PLET1^{+}$ TEC in the thymic epithelial differentiation hierarchy, which can together generate a fully functional thymus. No information yet exists regarding the reversibility or otherwise of the early steps in thymic epithelial lineage differentiation, and therefore other alternative explanations for these findings are also possible.

Taken together, the available genetic and functional analyses strongly support PLET1 as a marker of the founder cells of the thymic epithelial lineage and demonstrate the loss of PLET1 expression with the onset of differentiation in the fetal thymus [41]. The lineal relationship between fetal $PLET1^{+}$ TEPC and the population of $PLET1^{+}$ mTEC of the postnatal thymus remains unclear. While the $PLET1^{+}$ TEC population isolated from the fetal thymus at stages up to and including E16.5 could initiate de novo thymus organogenesis upon ectopic transplantation, this capacity was lost by E18.5 [178,179], indicating a clear functional difference between the early fetal and late fetal/postnatal $PLET1^{+}$ TEC populations. However, recent clonal resolution data indicate that a minor subpopulation of $PLET1^{+}$ cells in the adult thymus, that coexpresses the cortical marker LY-51, contains bipotent progenitor or stem cells that can generate both cTEC and mTEC in a transplantation assay [110]. These cells are thus likely to play a role in maintaining postnatal TEC, functioning either as adult common or mTEC-restricted TEC progenitor/stem cells [110] (discussed in sections that follow). Irrespective of the differences between fetal and adult populations, the identification of the earliest fetal progenitor cells for the TEC lineage [41,105,106,173], coupled with the demonstration that these cells are common TEPC [108] and can generate an organized, functional thymus upon transplantation [105,106], points to the possibility of using TEPC as a source of TEC in the generation of in vitro thymus organoids and in thymus transplantation.

The existence of intermediate mTEC- and cTEC-restricted progenitors in the fetal and postnatal thymus has now been demonstrated by a number of studies. Analysis of MHC Class II mismatched chimeras showed

that the medullary epithelium initially forms as individual clonally derived islets, and that these coalesce later in development [104]. This study demonstrated the presence of mTEC sublineage-restricted progenitors (mTEPC) until at least E15.5 [104]. Furthermore, medullary sublineage-restricted TEPC can be isolated from E13.5 fetal thymus based on expression of Claudin 3 (CLDN3) and CLDN4 [180], and the UEA1$^+$ subset of fetal CLDN3,4$^+$ TEC was shown to represent the precursor of the clinically important AIRE$^+$ subset of mTECs [180]. Further investigation of the fetal CLDN3,4$^+$ population has shown that robust mTEC generation can initiate from an SSEA-1$^+$ subset that lacks the expression of differentiation markers such as UEA-1, MHCII, and CD40 [181]. This population also persists in the adult thymus, although its capacity for mTEC generation is significantly diminished compared to its fetal counterpart [181]. A cTEC progenitor activity has also been identified within the CD205 TEC population of the fetal thymus [47], the descendants of which are suggested to acquire more differentiated characters in a stepwise fashion. CD205 is a broad marker of cTEC in the postnatal thymus and identification of cTEC progenitors within this population is therefore an area of current investigation.

The studies described above demonstrate that TEC development during ontogeny is driven by bipotent TEPCs that give rise to distinct precursor populations that are restricted to the cTEC and mTEC lineages. Further work is needed to phenotypically define the progenitor state, or states, at which the transition from bipotent TEPC to cTEC or mTEC-restricted progenitor occurs, and the molecular mechanisms controlling this transition. In this regard, CD205$^+$ TEPC populations isolated from E15.5 thymi can generate both cTEC and mTEC lineages [182]. Consistent with this observation, mTECs have been shown to derive from cell that have previously expressed the thymus-specific proteasome subunit β5t [183–185] and the TSCOT promoter [186] and strongly suggest that in the adult thymus mTEC is maintained by sublineage restricted rather than common or bipotent progenitors [184,185].

Human thymus development

Early human thymus development closely parallels that of the mouse; the human thymus forms from the 3PP in a common primordium with the parathyroid gland. The 3PP is evident from early week 6 of human fetal development and initially develops as a tube-like lateral expansion from the pharynx, which makes contact with the ectoderm of the third pharyngeal cleft [187,188]. Since the thymus has a single endodermal origin in mice and avians [138,164] it is reasonable to assume that this is also the case in humans, although this has not formally been demonstrated. Within the human common thymus/parathyroid primordia the thymus and parathyroid domains are located ventrally and dorsally and are surrounded by condensing NCC-derived mesenchyme from the onset of development [187]. The thymus component of this primordium begins to migrate ventrally from week 7 to midweek 8, forming a highly lobulated, elongated, cord like structure. The upper part of this structure normally disappears at separation of the two organ rudiments, leaving the parathyroid in the approximate location in which it will remain throughout adulthood [187]. The bilateral thymic primordia continue to migrate toward the midline, where they eventually meet and attach at the pericardium—the permanent location of the thymus into adulthood—by midweek 8 [187]. As in the mouse [105], the human early thymus primordium appears to contain undifferentiated epithelial cells that express some markers that are later restricted to either cortical or medullary compartments [169]. Nascent medullary development is evident from week 8, and by week 16, distinct cortical and medullary compartments are present. Other cell types penetrate the thymus from week 8, including mesenchymal, vascular, and lymphoid cells, and mature lymphocytes begin to leave the thymus to seed the peripheral immune tissues between weeks 14 and 16 [189,190].

Cervical thymus in mouse and human

In addition to the thoracic thymus, that is the main site of T cell production, the presence of an additional "cervical" thymus, located anterior to the thoracic thymus, has been recorded in both animals [191] and humans [189,192,193] and is a common occurrence in at least some mouse strains [194,195]. In terms of size and cellularity the cervical thymus is much smaller than the thoracic thymus; however, the morphology of the two structures is very similar, with organized cortical and medullary regions and similar expression patterns of cytokeratin molecules [194,195]. Furthermore, the cervical thymus expresses the transcription factors FOXN1 and AIRE and can produce functional T cells that are tolerant to self-antigens [157,195], although the range of self-antigens present may be more restricted than in the thoracic thymus [194].

In mice, cervical thymi are thought to appear at around E15.5 based on *Foxn1*-reporter expression [196,197]. Their frequency increases through late gestation, leveling off at birth at about 50% of mice on the C57BL/6 background, suggesting that cervical thymi all arise at or before birth [196], similar to humans where cervical thymi are clearly present in the second trimester of fetal development. The origin of the cervical thymus has been investigated in mice using both genetic and lineage tracing analyses [196]. Cervical thymi were shown to have two lineage origins. The majority appear to arise

from 3PP cells at the thymus-parathyroid junction, which have not previously differentiated into either organ fate, and turn on *Foxn1* expression after E15.5: as cells specified to the TE lineage retain their identity in the absence of FOXN1 expression [174,175], many of these cervical thymi could arise from FOXN1-negative but thymus-fated cells. About 25% of cervical thymi have a lineage history of parathyroid cell differentiation and appear to have downregulated the parathyroid program and switched to thymus [196]. TEC in parathyroid-derived cervical thymi express lower levels of *Foxn1* than do thoracic TEC and exhibit low levels of MHC Class II and absent UEA-1. As a result, parathyroid-derived cervical thymi support a distinct thymocyte differentiation profile, similar to other *Foxn1* hypomorphic thymi [198]. Thus cervical thymi with different lineage histories generate distinct sets of thymocytes and could contribute differentially to the peripheral T cell pool.

Molecular regulation of thymus and parathyroid organogenesis

Although the regulation of thymus organogenesis is incompletely understood, studies of classical and genetically engineered mouse mutants have revealed a network of transcription factors and signaling molecules that act in the pharyngeal endoderm and surrounding mesenchyme and mesoderm to regulate thymus and parathyroid organogenesis. The principal components of this network are discussed later and summarized in Fig. 37.2.

Improved understanding of molecular regulation of early TEC development may facilitate directed differentiation of pluripotent stem cells into TEC in vitro. Indeed, by recapitulating the stepwise restriction of differentiation potential observed in vivo, a highly enriched anterior foregut endoderm population can be obtained from pluripotent stem cell–derived definitive endoderm [199–204]. Some progress toward generating TEC from pluripotent stem cells has been reported [205–210], although production from pluripotent stem cells of TEC that can contribute to all normal TEC subtypes has not yet been reported [211].

Molecular control of early organogenesis

The T-box transcription factor TBX1, retinoic acid (RA) signaling, and fibroblast growth factor 8 (FGFf8) signaling have been implicated as important regulators of the earliest events in thymus organogenesis, which occur prior to overt organ development and relate to molecular control of 3PP formation.

Tbx1 is the gene responsible for cardiovascular and glandular defects in *Df1* mice, which carry a large deletion of chromosome 16 [212]. *Df1* heterozygotes closely phenocopy a human condition known as 22q11.2 deletion syndrome (22q11.2DS, or DiGeorge Syndrome) in which a deletion in chromosome 22 covering an interval of approximately 30 genes [213], results in a range of defects including thymus aplasia or, more frequently, hypoplasia [214,215].

During development, *Tbx1* is expressed in the pharyngeal endoderm and the core mesenchyme of the pharyngeal arches from approximately E7.5 and continues to be expressed in a variety of structures until E12.5 [216–218]. *Tbx1* mutants have severe defects throughout the pharyngeal region, including abnormal patterning of the first pharyngeal arch, hypoplasia of the second arch, and absence of the third and fourth arches and pouches [219]. As a result, $Tbx1^{-/-}$ mutants lack both thymus and parathyroids and display a spectrum of cardiovascular abnormalities and craniofacial defects [219].

The phenotype of $Tbx1^{-/-}$ animals suggests an important role in the segmentation of the pharyngeal region. Support for this hypothesis was provided by an elegant study addressing the temporal requirement for *Tbx1* in the development of the pharyngeal region. Deletion of *Tbx1* at E8.5, during the formation of the 3PP, resulted in complete absence of thymus and parathyroid, and complementary fate-mapping experiments demonstrated that cells that express *Tbx1* at E8.5 contribute significantly to the thymic primordium [220]. However, although deletion of *Tbx1* at E9.5/E10.5 (after initial formation of the 3PP) caused morphological defects in the thymus, these were not as severe as the aplasia seen after deletion at E8.5, and fate mapping of cells expressing *Tbx1* at E9.5/E10.5 revealed only a small contribution to the thymus [220]. This is likely because *Tbx1* is downregulated in the ventral 3PP during pouch outgrowth [221,222] and, in fact, there is evidence that its expression is antagonistic to thymus differentiation. Ectopic expression of *Tbx1* in *Foxn1*-expressing cells results in *Foxn1* downregulation, with the cells remaining $PLET1^{+}$ and presumably thymus-fated [222]. Taken together, these data suggest that TBX1 is required for the formation of the 3PP but is not directly required for subsequent thymus development.

A role for RA in 3PP formation was suggested by experiments in which RA antagonist was administered to whole embryo cultures. Blockade of RA signaling at E8.0 resulted in the absence of the growth factors FGF8 and FGF3 in the 3PP endoderm and impaired NCC migration to the third and fourth pharyngeal arches [223]. Expression of the transcription factor PAX9 (see later) was also absent in the 3PP but was expanded in the 2PP endoderm. These data suggest that RA signaling is required for the specification of the 3PP, which confers subsequent competence to support NCC migration. Genetic evidence for a role for RA signaling was subsequently provided by the finding that fetal mice lacking RA receptors α and β display thymus agenesis and

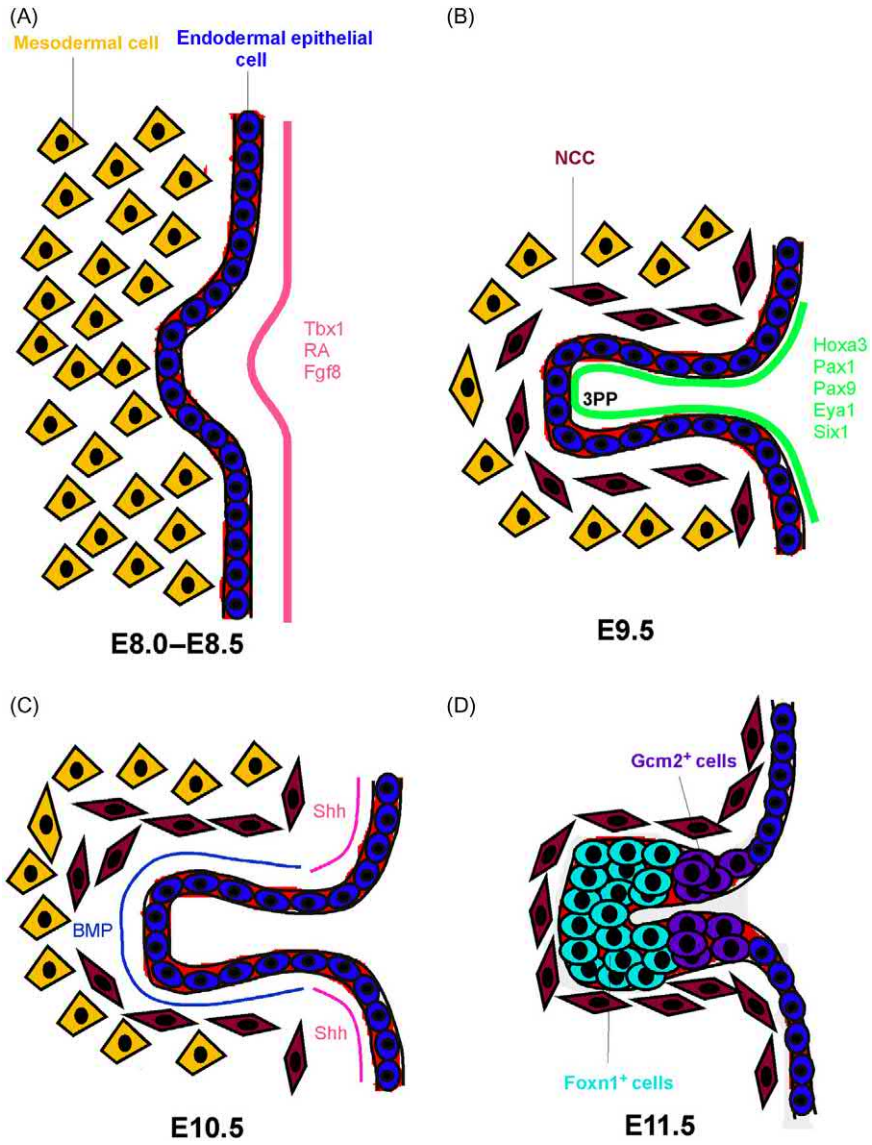

FIGURE 37.2 Molecular regulation of early thymus organogenesis.
(A) At approximately E8.0−E8.5 the formation of the 3PP is initiated in the pharyngeal endoderm and is dependent on the expression of TBX1 and RA, and on FGF8 signaling (pink). (B) At E9.5 the 3PP has formed and is surrounded by mesenchymal cells of mesodermal and NCC origin. Continued development is dependent on the expression of the transcription factors HOXA3, PAX1, PAX9, EYA1, and SIX1 (green). (C) BMP (blue) and SHH (pink) signaling occur at E10.5 in the 3PP endoderm in the ventral and dorsal aspects, respectively. These factors may be involved in the specification of the 3PP into thymus and parathyroid specific domains. (D) At E11.5 epithelial cells in the ventral domain of the 3PP express the transcription factor FOXN1 (light blue) and will form the thymic epithelium. Epithelial cells in the dorsal domain express the transcription factor GCM2 (purple) and will form the parathyroid gland. The differentiation and maintenance of both of these cell types are dependent on these factors. Note that throughout this figure, gene-expression domains are annotated only for the endoderm. Many of the factors shown are also expressed in the mesoderm and/or mesenchyme of the pharyngeal arches, and/or in the pharyngeal ectoderm, as described in the text. *3PP*, third pharyngeal pouch; *BMP*, Bone Morphogenetic Protein; *FGF8*, fibroblast growth factor 8; *NCC*, neural crest cell; *RA*, retinoic acid; *SHH*, sonic hedgehog.

ectopia [224]. Furthermore, exposure to excess RA also resulted in defects in third and fourth PP formation, altered NCC migration, and subsequent thymus development defects, which were associated with altered *Hoxa3* and *Pax1* expression [225].

FGF8 is also required during the early stages of thymus and parathyroid development. This growth factor is expressed in the early gut endoderm and in the endoderm and ectoderm of the pharyngeal pouches and clefts. Mice carrying hypomorphic alleles of *Fgf8* show defects in thymus development ranging from hypoplasia to complete aplasia [226,227]: the initial impairment in thymus and parathyroid organogenesis is likely to occur at an early stage in the development in these mice, as the third and fourth pharyngeal arches and pouches are usually hypoplastic/aplastic, in addition to other abnormalities in the pharyngeal region [226,227].

In terms of the cell types affected by impaired FGF8 signaling, similarities between the phenotype of FGF8 hypomorphs and the spectrum of abnormalities found during experimental NCC ablation [228,229] suggest that the glandular defects may result from defective NCC migration/differentiation or survival. In support of this, NCC of FGF8 hypomorphs show increased levels of apoptosis [226,227] and reduced expression of *Fgf10*, which mediates the expansion of the thymic primordium from E12.5 [154], in the neighboring NCC mesenchyme [227]. There is also a mild reduction in the expression of genes associated with differentiated NCC [226] suggesting that the maintenance of NCC is perturbed.

Taken together, these data implicate FGF8 in maintaining a competent NCC population that can contribute to thymus organogenesis. However, FGF8 may also act specifically on the 3PP endoderm, since ablation of FGF8

in the endoderm and ectoderm, or ectoderm alone, results in different phenotypes. Ablation in the ectoderm alone causes vascular and craniofacial defects as seen in FGF8 hypomorphs [230], whereas when FGF8 is also deleted in the endoderm, glandular defects are evident, including thymus hypoplasia and ectopia [230]. The NCC defects were the same in both cases.

Further insight into the role of FGF signaling was provided by a detailed study of FGF signaling components during thymus organogenesis. This revealed a complex picture in which FGF signaling is modulated by the expression of Sprouty 1 and 2, such that although the 3PP expresses both FGFs and FGFR, signaling in the endodermal component of the thymus is restricted by Sprouty 1 and 2 [231]. Thus while the importance of FGF8 signaling in regulating development of the 3PP is clear, further studies are required to understand the temporal requirements for this pathway and its interplay with other signaling pathways involved in thymus and parathyroid organogenesis.

Transcription factors and regulation of third pharyngeal pouch outgrowth

After initial 3PP formation, continued development of the common primordium is dependent on a complex networks of transcription factors, including HOXA3, PAX1, PAX9, EYA1, SIX1 and SIX4; all of these factors are expressed in the 3PP endoderm from E9.5 to E10.5 and, with the exception of PAX1 and PAX9, are also expressed in associated NCC and ectoderm.

Hoxa3 mutants are both athymic and a parathyroid [145,232], and analysis of both null and tissue specific null mutants together reveals a complex role for HOXA3 in thymus development [233]. Initial 3PP formation is normal in *Hoxa3* null mutants, but the location and timing of regionalized markers including *Bmp4, Tbx1*, and *FGF8* is altered. *Gcm2* is initially expressed in the correct parathyroid domain but is then downregulated with the cells undergoing apoptosis, consistent with the *Gcm2* null phenotype [234]. In the thymus domain of $Hoxa3^{-/-}$ mice, *Foxn1* expression is delayed but subsequently reaches normal expression levels; however, the entire thymus primordium undergoes rapid apoptosis soon after and is completely ablated by E12.5. Conditional deletion in the endoderm or NCC or both showed that expression in each tissue contributes uniquely to thymus development, and that expression in either tissue is sufficient for thymus survival. A further study using temporally controlled global *Hoxa3* deletion showed that *Hoxa3* is not required in the thymus after E12.5 [233]. Thus HOXA3 function is required in both NCC and endoderm for proper timing of thymus differentiation, and its expression in either cell type is sufficient for survival of the thymus primordium.

Deletion of *Eya1* results in the failure to initiate overt thymus and parathyroid organogenesis once the 3PP has formed, revealing the essential roles of these factors [144,145,235,236]. *Tbx1* and *Fgf8* are both downregulated in the 3PP of $Eya1^{-/-}$ mice at E9.5 [236], indicating that EYA1 plays a role in regulation of these factors. Lack of SIX1 and SIX4, PAX1, or PAX9 causes much less severe phenotypes. The common primordium begins to develop in $Six1^{-/-}$ mice, and patterning into thymus and parathyroid domains (see the "Specification of the thymus and parathyroid" section) is initiated. However, subsequent apoptosis of endodermally derived cells in the common primordium leads to complete disappearance of the organ rudiment by E12.5 [236]. A similar phenotype is evident in $Six1^{-/-}$; $Six4^{-/-}$ embryos, though the size of the primordium is further diminished in the double mutants indicating synergy between these gene products [236]. *Pax9* loss of function mutations result in the formation of ectopically located thymic primordia, which form within the laryngeal cavity, rather than within the pharyngeal arch mesoderm. These primordia fail to migrate to the mediastinum and exhibit severe hypoplasia from E14.5. However, the thymic lobes are vascularized, contain both thymocytes and mesenchymal cells, and express *Foxn1* [237]. $Pax1^{-/-}$ mutants show relatively mild thymus hypoplasia and aberrant TEC differentiation [146,238,239]. Since *Pax1* and *Pax9* are highly homologous, these phenotypes may reflect functional redundancy, as demonstrated in other tissues [240,241].

Expression of *Hoxa3*, *Eya1*, and *Six1* in multiple germ layers complicates interpretation of the respective null phenotypes for these genes. However, HOXA3 appears to regulate *Pax1* and *Pax9* either directly or indirectly, since *Pax1* and *Pax9* expressions are initiated normally in $Hoxa3^{-/-}$ mutants but fails to be maintained at wild-type levels beyond E10.5 [145]. Furthermore, $Hoxa3^{+/-}$; $Pax1^{-/-}$ compound mutants show delayed separation of the thymus/parathyroid primordium from the pharynx, resulting in thymic ectopia and a more severe hypoplasia than that seen in $Pax1^{-/-}$ single mutants [146], indicating that these factors function in the same network.

It has been suggested that EYA1 and SIX1 act downstream of the *Hox/Pax* genes, as $Eya1^{-/-}$ embryos show normal expression of *Hoxa3*, *Pax1*, and *Pax9* but reduced expression of *Six1* in the endoderm of the third and fourth PP and ectoderm of the second, third, and fourth pharyngeal arches [235]. However, while it is likely that SIX1 acts downstream of EYA1, more recent evidence indicates that EYA1 and SIX1 are not regulated by the PAX genes since $Pax9^{-/-}$ and $Pax1^{-/-}Pax9^{-/-}$ mutants show normal expression of *Eya1* and *Six1* in the 3PP. Interestingly, $Eya1^{-/-}$ and $Six1^{-/-}$ double mutants lack the expression of *Pax1* in the E10.5 3PP, while *Pax9* expression is unaffected [236]. It remains possible that

Eya1 and *Six1* may be regulated by HOXA3 independently of PAX1 and PAX9 function.

Mice homozygous for a null mutation in *Ripply3*, an RA signaling target, have ectopic and hypoplastic thymi, with the thymic lobes of *Ripply3*$^{-/-}$ embryos being located in the oropharynx, similar to *Pax9* null thymi [242,243]. *Ripply3* was shown to repress *Tbx1* transcription in an in vitro Luciferase assay system, and the expression domain of *Pax9* was increased in the pharyngeal region of E9.5–E10.5 *Ripply3*$^{-/-}$ mice [242]. Furthermore, a 3.7 kb promoter region of *Pax9* was shown to be TBX1 responsive in a Luciferase assay. These studies together suggest that the expression of *Tbx1* may regulate *Pax9* expression.

Overall, the complexity of these data suggests it is likely that all or some of these factors form a stable network, which is sustained by complex feedback and autoregulatory loops. Further work is required to elucidate exactly how these and other transcription regulators cooperate in the development of the 3PP endoderm and to determine the precise role of each of these factors at the cellular level.

Specification of the thymus and parathyroid

Prior to the overt the formation of the thymus and parathyroid primordia, the 3PP is specified into organ-specific domains. At E9.5, epithelial cells within the anterior dorsal aspect initiate expression of GCM2 [244], a transcription factor required for the development of the parathyroid [245]. *Gcm2* is downregulated in *Hoxa3*$^{-/-}$, *Eya1*$^{-/-}$ and *Hoxa3*$^{+/-}$;*Pax1*$^{-/-}$ compound mutant mice [146,165,235], suggesting that GCM2 acts downstream of EYA1 and HOXA3. *Gcm2* is also directly regulated by GATA3; in E11.5 *Gata3*$^{+/-}$ mice, a lower proportion of cells in the 3PP expresses *Gcm2* [246]. The first known thymus specific transcription factor, FOXN1, is expressed at functionally relevant levels in the ventral domain of the 3PP from approximately E11.25, although low levels can be detected from E10.5 [244,247]. FOXN1, a forkhead class transcription factor, is required for TEC differentiation and hair development, and is discussed in more detail in the "FOXN1 and regulation of thymic epithelial cell differentiation" section.

The expression patterns of *Foxn1* and *Gcm2* clearly define the thymus and parathyroid domains of the 3PP. However, these factors do not appear to be responsible for specification of their respective organs, indicating that specification must be mediated by an upstream factor or factors. Three lines of evidence support this model. First, the epithelial cells in the ventral domain of the E11.5 3PP express interleukin 7 (IL-7), a cytokine required for thymocyte differentiation [248], making IL-7 one of the earliest currently identified markers of thymus identity. The thymus primordium *Foxn1*$^{-/-}$ embryos show normal *IL-7* expression [248], indicating that TE identity is specified in the absence of FOXN1. Second, the transplantation of the E9.0 pharyngeal endoderm, which does not express functionally relevant levels of *Foxn1*, gives rise to a functional thymus when grafted ectopically [138], indicating that at this developmental stage, some pharyngeal endoderm/3PP cells are already specified to the TE lineage. Third, the 3PP cells of mice carrying revertible null or revertible hypomorphic alleles of *Foxn1* retain their identity as TEPC until at least postnatal day 14 and 6 months of age, respectively, and are induced to initiate the TEC differentiation/organogenesis program simply by reversion of the null or hypomorphic allele [174,175].

Similarly, our studies conclude that GCM2 is not required for the specification of the parathyroid, as other parathyroid-specific markers, including *Tbx1*, *Ccl21*, and *CaSR*, are initiated in *Gcm2*$^{-/-}$ mice, although this domain undergoes rapid and coordinated apoptosis in the absence of GCM2 [249]. Thus GCM2 may play a somewhat analogous role in parathyroid development to that of FOXN1 in the thymus, although the thymus rudiment does survive in the absence of *Foxn1*.

Upstream regulation of *Foxn1* is currently not well understood. *Eya1*$^{-/-}$ embryos do not express *Foxn1*, but this is likely due to the block in primordium formation prior to the onset of *Foxn1* expression in each of these mutants. Although *Foxn1* expression is unaltered in *Pax9*$^{-/-}$and *Hoxa3*$^{+/-}$, *Pax1*$^{-/-}$ mutants [237,239], PAX1 and PAX9, are known to be functionally redundant in other cell-types, as expected from their high homology level. Furthermore, while *Six1*$^{-/-}$ mutants display reduced *Foxn1* expression, the 3PP exhibits increased cell death in the absence of *Six1* [236], and therefore, the reduced expression of *Foxn1* may reflect poor survival of FOXN1^{+} cells rather than a direct interaction between *Foxn1* and SIX1. Further studies are required to determine whether a regulatory relationship exists between these genes and *Foxn1*.

Several candidate regulators of thymus specification have been identified based on their expression in the 3PP prior to initiation of *Foxn1* [250]. These include NKX2.5, NKX2.6, ISL1, GATA3, and FOXG1. *Nkx2.5*, *Nkx2.6*, *Isl1*, and *Gata3* are expressed in the developing 3PP at E9.5 and are restricted to the ventral portion of the 3PP endoderm at E10.5. *Foxg1* is not expressed in the 3PP at E9.5 but at E10.5 is expressed in two discrete regions, one dorsal and the other ventral. By E11.5, when *Foxn1* is first activated in the 3PP, *Foxg1* and *Isl1* are already restricted to the thymus domain. These factors continue to be expressed in TECs throughout late fetal and postnatal differentiation. However, it remains unknown whether they transcriptionally activate *Foxn1* or instead regulate

FOXN1-independent aspects of thymus development. The transcription factors E2F3 and E2F4 have been shown to play a role in promoting FOXN1 expression in the adult thymus [251]. E2F family transcription factors are associated with cell cycle progression and are negatively regulated by Retinoblastoma (RB) tumor suppressor proteins. Both E2F3 and E2F4 have been shown to bind to their consensus motifs in the presumptive FOXN1 promoter and genetic ablation of the Retinoblastoma tumor suppressor proteins in adult mice results in increased E2F3 activity and a concomitant increase in FOXN1 expression in TEC [251]. As a result of perturbed RB activity, these mice exhibit thymic hyperplasia, which can be reversed by genetically suppressing FOXN1, strongly suggesting that the effects observed reflect E2F mediated regulation of FOXN1.

The T-box transcription factor TBX1 has also been implicated in the regulation of FOXN1 expression. As discussed above, overexpression of TBX1 in 3PP in mice leads to reduced FOXN1 expression and maturational arrest of TEC differentiation at an early progenitor stage, characterized by expression of PLET1 [222]. This indicates that TBX1 negatively regulates FOXN1 expression, although further work is required to establish if this effect is direct or indirect.

Further insight regarding the regulation of *Foxn1* expression may be drawn from analysis of the cutaneous epithelium, which also requires FOXN1 for normal development. There is some evidence to suggest that HOXC13 is involved in *Foxn1* regulation in hair follicles [252], although at present it has not been determined if this factor performs a similar function in TEC.

How then are the thymus and parathyroid domains within the 3PP established? Evidence suggests that opposing gradients of bone morphogenetic proteins (BMPs) and sonic hedgehog (SHH) may play an important role in this process. During thymus development, *Bmp4* expression is first detected at E9.5 when it is expressed by a small number of mesenchymal cells in the third pharyngeal arch [253]. By E10.5, the *Bmp4* expression domain has expanded to include the ventral 3PP endoderm and the adjacent mesenchyme but remains absent from the dorsal 3PP. This expression pattern is maintained at E11.5 and by E12.5 *Bmp4* is expressed throughout the thymic primordium and the surrounding mesenchymal capsule [253].

In vivo evidence of a role for BMPs in thymus organogenesis was provided by a series of studies in which the expression of the BMP inhibitor *Noggin* was driven by the *Foxn1* promoter, thus impairing BMP signaling in the thymic stroma [254–256]. *Foxn1-Noggin* mice developed hypoplastic and cystic thymi that failed to migrate to their normal position above the heart. It is highly likely that this is due to a direct effect on the thymic stroma, although mediators of BMP signaling such as *Msx1* and phosphorylated SMAD proteins were downregulated in both the epithelium and surrounding mesenchyme. Interestingly, *Foxn1* expression was only partially blocked in this transgenic model [254]. However, *IL7* expression was also absent in the cells that had downregulated *Foxn1*, suggesting that thymus fate, rather than solely *Foxn1*, may have been affected [255]. A caveat for interpreting these data is that the inhibition of BMP signaling was driven by the *Foxn1* promoter and thus occurred after initiation of *Foxn1* expression. Furthermore, the addition of a BMP inhibitor did cause loss of *Foxn1* in a zebra fish model [255]. A further study showed that BMP signaling is necessary for *Foxn1* initiation in cocultures of qE2.5 quail pharyngeal pouch endoderm and chick mesenchyme. BMP is required only during a narrow time window, however, as addition of Noggin has no effect on *Foxn1* initiation in cocultures containing qE3 endoderm. *Gcm2*, in contrast to *Foxn1*, is initiated in the absence of mesenchyme and maintained in the presence of Noggin [257]. Furthermore, transient inhibition of BMP signaling during early/mid thymus organogenesis led to reduced *Foxn1* expression, with concomitant reduced functionality also observed in $Foxn1^{+/-}$ TEC [256].

Conditional deletion of *Bmp4* in the pharyngeal endoderm (and, with variable efficiency, in the surrounding mesenchyme) using *Foxg1-Cre* indicates that expression of *Bmp4* is required for thymus and parathyroid morphogenesis. In E11.5 *Foxg1-Cre*; $Bmp4^{F/F}$ embryos, the thymus-parathyroid primordium is hypoplastic, but the expression of *Foxn1* and *Gcm2* is normal as detected by in situ hybridization, demonstrating proper specification and patterning of the thymus and parathyroid domains. However, delayed separation of the two organ primordia and reduced migration of the thymus primordia are evident at E12.5 and E13.5 [258]. *Wnt1-Cre* or *Foxn1-Cre* mediated the deletion of *Bmp4* has no effect on thymus and parathyroid morphogenesis, suggesting that BMP4 signaling is required in the pharyngeal epithelium during a narrow time window prior to the onset of *Foxn1* expression. However, as the thymic primordium expresses BMP2 and BMP7 (CCB and NRM unpublished data), which signal through the same receptor as BMP4, redundancy is an issue for interpreting these results.

During the development of the 3PP, expression of the secreted glycoprotein SHH is restricted to the region of 3PP immediately adjacent to the pharyngeal endoderm at E10.5 and E11.5, although its receptor Patched1 is expressed by cells in close proximity to this region [259,260]. Analysis of $Shh^{-/-}$ embryos revealed expanded expression domains for both *Bmp4* and *Foxn1* in the 3PP, while the corresponding $Gcm2^{+}$ parathyroid domain was lost in these mutants [259]. Consistent with these observations, cyclopamine treatment of chick embryos at HH14–16 blocked *Gcm2* initiation and led to

enhanced *Bmp4* expression in the caudal pharyngeal pouches. However, loss of SHH signaling at a later stage (HH21) caused ectopic expression of *Gcm2* and *CaSR* in the anterior first ectodermal cleft and posterior second arch and had no effect on *Gcm2* transcription in the 3PP [260]. Thus at early developmental stages, the role of SHH in thymus organogenesis may be to oppose the action of BMP4 to allow the specification and development of the parathyroid. At later stages, SHH signaling may also restrict parathyroid development to the caudal pharyngeal pouches.

SHH is also implicated in both thymocyte and TEC development [261]. In the mouse, $Shh^{-/-}$ results in embryonic lethality at E15.5. Analysis of thymic cellularity at E15.5 revealed a decrease in both the EPCAM and CD45 expressing compartments and, when cultured as FTOC for 7 days, displayed a lower mTEC to cTEC ratio than $Shh^{+/+}$ controls. To isolate the effect of SHH on TEC development from the above-described effect upon 3PP specification, the authors employed a conditional SHH knockout model, *Foxn1-Cre*; $Shh^{F/F}$, in which *Shh* is only deleted in *Foxn1* expressing TEC (and skin). At E15.5 the model displayed reduced total thymic and TEC cellularity compared to *Cre*-controls. When cultured in FTOC, similar perturbation of the medullary compartment was observed as in the constitutive *Shh* knockout model. Examination of maturity markers in conditional *Shh* knockout TEC showed that mTEC expressed significantly more MHCII but that fewer cells developed AIRE expression. Collectively, these data indicate that SHH is required for 3PP specification and is antagonistic to thymus development during initial organogenesis but has subsequent effects on TEC development.

WNT glycoproteins may also be important in regulating *Foxn1* expression. WNTs are expressed by the thymic stroma and by lymphoid cells, with WNT receptors being expressed by TECs [247] and some other intrathymic cell-types including DCs. The earliest reported expression of WNT family members during thymus development is at E10.5, immediately prior to strong *Foxn1* expression, when the epithelium of the 3PP and adjacent cells express *Wnt4* [247]. Given this expression pattern and the finding that TEC lines that overexpress WNT4 display elevated the levels of *Foxn1* [247], it is possible that WNT4 cooperates with BMP4 to regulate *Foxn1*. However in vivo models fail to provide evidence of this. $Wnt1^{-/-}$, $Wnt4^{-/-}$, $Wnt1^{-/-}$, *and* $Wnt4^{-/-}$ mice all exhibit hypoplastic thymi characterized by reduced T cell numbers but normal thymocyte developmental progression, indicating that *Foxn1* is activated in the absence of these WNT signaling cascades [107,262].

Analysis of $Wnt4^{-/-}$ mice suggests that WNT4 controls thymus size by modulating the expansion of both TECs and immature thymocytes. By E15.5, $Wnt4^{-/-}$ thymi contained fewer TECs than wild-type thymi, and at E18.5, histological analysis revealed smaller medullary areas and reduced the expression of medullary markers and disorganization of the CMJ. In addition, mouse models have been engineered to knock out the canonical, β-catenin-dependent and noncanonical, Nlk-dependent ($Nlk^{-/-}$) intracellular signaling cascades [263], with TEC- and skin-specific knockout of β-catenin (*Foxn1*-Cre; $Ctnnb^{fl/fl}$) resulting in neonatal lethality. At E15.5, both models led to hypocellular thymi, similar to $Wnt4^{-/-}$ mice but with histologically normal TEC patterning and robust *Foxn1* expression. The authors also explored the effects of WNT overactivation in TEC using *Foxn1*-driven expression of β-catenin (*Foxn1:Ctnnb*) and WNT4 (*Foxn1:Wnt4*). In both models, thymic organogenesis was severely perturbed, characterized by ectopic, and hypocellular thymi with reduced *Foxn1* expression compared to wild-type controls. In the β-catenin overexpression model, TEC were unable to form a reticulated architecture, the thymus was not colonized by T cells, and adult mice were severely T cell hypoplastic [263]. The WNT4 overexpression model produced comparatively larger thymi with reticulated TEC architecture but still presented disrupted differentiation [263]. These observations are consistent with a previous analysis, that also showed restricted immigration of thymocytes, as well as a block in thymocyte development at the DN1 stage, in a β-catenin overexpression model [264]. The mutant TE contained a central core of cells that initially appeared committed to a thymus fate but later downregulated expression of FOXN1, K8 and K5, and expressed involucrin [264]. Similarly, the deletion of the WNT signaling repressor *adenomatous polyposis coli* (*Apc*) in K14-expressing cells (using *K14-Cre*; $Apc^{CKO/CKO}$ mice) revealed a hypoplastic thymus characterized by variable disruption of epithelial organization, exclusion of lymphocytes, and the presence of enlarged structures expressing keratinocyte markers such as involucrin [265].

Collectively, these data suggest that WNT acts as a negative regulator of *Foxn1* and inhibits migration of the thymic rudiment. Analysis of a double transgenic model in which both β-catenin and the BMP4 inhibitor NOGGIN were overexpressed in TEC (*Foxn1:Ctnnb*; *Foxn1:Noggin)* revealed that the inhibition of BMP4 further exacerbated the β-catenin overexpression phenotype. However, later in development WNT appears essential for thymic organogenesis as its absence perturbs thymic cellularity and TEC differentiation [263].

Evidence also suggests that WNT signaling is also required for the maintenance of the postnatal thymus. Deletion of the WNT antagonist Kremen resulted in the development of large epithelial-free zones in the TEC network [266]. Tetracycline-regulated the overexpression of the canonical WNT inhibitor Dickkopf related protein 1

(encoded by *Dkk1*) in cortical, and medullary TECs of postnatal mice resulted in dramatic thymus degeneration [267]. A decrease in the number of $K5^{+}K8^{+}$ cells at the CMJ and reduced proliferation in the MHC Class II^{lo} compartment was observed in this model, perhaps implicating WNT in the regulation of progenitor TEC. Consistent with this idea, these phenotypes were reversed by the withdrawal of Doxycycline, and restoration of thymus size correlated with recovery of the $K5^{+}K8^{+}$ TEC population [267].

Foxn1 and regulation of thymic epithelial cell differentiation

As discussed in the "Origin of thymic epithelial cells" and "Thymic epithelial progenitor cells (TEPC)" sections, the existence of a common TEPC, able to generate both cortical and medullary TECs, and of downstream cortical and medullary thymic epithelial sublineage-restricted TEPC, is now clearly established. Understanding the mechanisms controlling TEC sublineage differentiation from the common progenitor, and the maintenance of differentiated TEC subtypes, is thus important for tissue-engineering strategies.

The transcription factor FOXN1, introduced in the "Thymic epithelial progenitor cells" section, regulates the differentiation of TEC in both the mTEC and cTEC sublineages in the fetal and adult thymus. TEC undergo developmental arrest at the founder/fetal progenitor cell stage of development in the absence of functional FOXN1 expression [41,174]. FOXN1 is required cell autonomously for development of all mature TEC subtypes from the common TEPC [170] and also regulates TEC proliferation in the fetal thymic rudiment [143]. Thus while TEPCs form independently of FOXN1, their proliferation and differentiation into mature c- and mTEC is FOXN1-dependent. However, analysis of the $Foxn1^{-/-}$ fetal thymus revealed the presence of $K5^{hi}CLDN4^{hi}$ regions corresponding to presumptive progenitor mTEC suggesting that, during organogenesis, FOXN1 is not required for divergence of the mTEC lineage from the common TEPC [41]. This was corroborated by the finding that $Cldn3,4^{hi}SSEA1^{+}$ mTEC stem cells could be identified in $Foxn1^{-/-}$ fetal thymi, albeit at reduced numbers compared to wild-type controls [268].

FOXN1 is expressed by most if not all differentiating and mature TECs throughout fetal development and, postnatally, is also expressed in most TECs throughout the lifespan [171]. $FOXN1^{lo/negative}$ populations arise in the postnatal thymus from TEC that have previously expressed FOXN1 [269,270] with this progressive postnatal downregulation of FOXN1 occurring first in cTEC, coincident with the onset of age-related thymic involution [269]. The role of FOXN1 beyond the fetal TEPC stage is incompletely understood. FOXN1 is required in a dosage-dependent manner to regulate multiple stages of differentiation in both the c- and mTEC sublineages in the fetal and adult thymus. In both sublineages, FOXN1 is required throughout differentiation, from exit from the earliest TEPC state to terminal differentiation of both cTEC and $AIRE^{+}$ mTEC [41]. FOXN1 also plays a role in regulating cross talk between developing TEC and thymocytes, which is required for the expansion and maturation of the medullary compartment [198,271].

Importantly, a subfunctional thymus can be generated at suboptimal levels of FOXN1 expression or function, indicating that full thymus function is generated only in a relatively narrow *Foxn1* dosage range [41,198,272–274]. This conclusion is also supported by the analysis of FOXN1 function in the postnatal thymus, which shows it is required in a dosage-sensitive manner to maintain thymus homeostasis [275], and that complete loss of either FOXN1 expression or FOXN1-expressing TEC postnatally results in loss of thymus function [197,276,277]. Furthermore, downregulation of FOXN1 expression in TEC is a key hallmark of thymic involution [278–280]. Restoration of FOXN1 expression is sufficient to reverse this process in aged mice [280], and the upregulation of FOXN1 is also observed in some models of thymus regeneration [270]. Collectively, these data highlight the importance of understanding the intrinsic and extrinsic mechanisms that regulate FOXN1 expression, and the downstream mechanisms regulated by FOXN1, in vivo.

As discussed above, little information exists regarding the transcriptional regulation of *Foxn1* expression. However, direct or indirect transcriptional targets of FOXN1 itself have been described in a number of studies [25,41,197,272,275,280–284]. Recently, high-confidence direct FOXN1 targets in postnatal cTEC have been identified by chromatin immunoprecipitation sequencing (ChIPseq) [284]. Since the set of FOXN1-regulated genes identified to date includes factors with diverse functions in thymus development and the TEC-mediated support of T cell development, FOXN1 can be considered a "master regulator" of TEC differentiation, rather than a regulator of a specific aspect of thymus development.

In addition to its expression in TEC, FOXN1 is also expressed in skin and hair follicle keratinocytes, where it regulates normal development. In these cutaneous epithelial lineage cells, FOXN1 is believed to regulate the balance between proliferation and differentiation [285–287]. However, while some parallels may exist between FOXN1 function in skin and thymus, FGF2, a known direct target of FOXN1 in cutaneous epithelium [287], is not expressed in TEC [41], suggesting FOXN1 may regulate different target genes in the cutaneous and thymic epithelial lineages.

Medullary development and expansion

The development of mature mTECs depends on the activation of the noncanonical NF-κB-signaling pathway that culminates in RELB activation. mTEC development is severely compromised in RELB deficient mice [288,289]. In addition, a range of medullary defects occurs in mice deficient in components upstream of RELB in the alternative NF-κB-activation pathway. Mice with a naturally occurring mutation in NF-κB-inducing kinase (*NIK*), and *Ikkα* knockout mice, display abnormal thymic architecture with small, hypocellular medullary regions associated with reduced *Aire* and TRA expression [290–292]. A similar phenotype is found in *TRAF6* knockout mice, indicating that the classical NF-κB-signaling pathway also plays a role in formation of functional medullary regions [293]. Interestingly, each of these mutant strains develops autoimmune manifestations consistent with a breakdown in the establishment of central tolerance due to a deficiency of $AIRE^+$ mTECs.

Signals from positively selected thymocytes play an important role in expanding the medullary region in adult thymi, a mechanism often referred to as lympho-epithelial cross talk. Small medullary regions containing sparse mTEC subsets are found when thymocyte development is blocked at the DP stage, as occurs in *TCRα* deficient mice or in mice lacking both MHC Class I and MHC Class II molecules [294,295]. Despite the paucity of mTECs, the medullary regions in these mice nevertheless express *Aire* and TRAs, indicating that mTEC expansion is affected to a greater extent than differentiation. Positively selected DP thymocytes differentiate into SP thymocytes that migrate into the medulla, and these SP cells express various tumor necrosis factor superfamily (TNFSF) ligands, including receptor activator of NF-κB ligand (RANKL), CD40 ligand (CD40L), lymphotoxin-α, and lymphotoxin-β [296–299]. Binding of these ligands to their receptors on mTEC activates NF-κB signaling, to promote mTEC proliferation and differentiation: ligand engagement of the TNFSF receptors activates NIK, which phosphorylates homodimers of the downstream kinase, Ikkα. Activated Ikkα in turn phosphorylates the C-terminal region of NF-κB2 (p100) leading to ubiquitin-dependent degradation and release of the N-terminal polypeptide, p52. The formation of RELB/p52 heterodimers permits shuttling of RELB from the cytoplasm into the nucleus where it functions as a transcriptional regulator [300].

Thus *RANK* or *RANKL* deficient mice have small, poorly developed medullary regions in which mTEC cellularity is decreased and *Aire* expression is reduced [298,301]. Targeted disruption of the *Ltbr* gene also results in disorganized medullary regions that contain reduced numbers of mTECs [299]. Signaling via the LTBR is required to upregulate RANK expression on fetal TECs, implying cooperation between these two TNFSF receptors in mTEC development [302]. Furthermore, it has been reported that LTBR signaling regulates *Fezf2* expression in mTECs [93], suggesting a possible mechanism by which this signaling cascade regulates self-tolerance. However, a subsequent report found that neither *Aire* nor *Fezf2* expession was altered by loss of LTBR signaling [303]. The reason for the discrepancy in these two reports is not clear.

Recently, two studies have indicated a hierarchy of intermediate progenitors specific for the mTEC (MEC) sublineage, based on detailed genetic analyses of NF-κB pathway components [268,304]. These studies strongly suggest that RANK signaling promotes two distinct stages of mTEC development, initially cooperating with LTBR to induce "pro-pMEC" ($UEA\text{-}1^+RANK^{lo}MHC\ II^{lo}CD24^{hi}$) from the putative $Cld3,4^{hi}SSEA\text{-}1^+$ mTEC stem cell via RELB [268,304] and subsequently stimulating "pro-pMEC" to differentiate into "pMEC" ($RANK^+UEA\text{-}1^+MHC\ II^{mid}CD80^-$) and then $AIRE^+$ MEC via TRAF6 [304]. HDAC3 has also emerged as an essential regulator of mTEC differentiation [305], and a role for STAT3 signaling has been demonstrated in mTEC expansion and maintenance [306,307]. Despite these advances, the molecular mechanisms operating in thymus organogenesis to govern the emergence of the earliest cTEC- and mTEC-restricted cells are not yet understood [180].

Interestingly, medullary region development is initiated in the fetal thymus prior to the appearance of SP thymocytes. This is at least partly due to the presence of lymphoid tissue inducer (LTi) cells that express RANKL, but not CD40L. RANKL signaling induces the generation of $AIRE^+$ mTECs before fetal thymocytes of the αβ TCR lineage have matured to the SP stage [301,308]. The early fetal thymus also contains thymocytes expressing invariant Vγ5 Vδ1 TCRs. These cells, which are present prior to the development of αβ lineage thymocytes, also express RANKL and play a role in the development of $AIRE^+$ mTECs [309]. In addition to αβTCR and γδTCR expressing thymocytes, group 3 innate lymphoid cells (ILC3) and invariant natural killer cells also express RANKL and, therefore, contribute to mTEC differentiation.

Maintenance and regeneration of thymic epithelial cells: Progenitor/stem cells in the adult thymus

The identity of the progenitor and/or stem cell populations that maintain postnatal TECs during homeostasis has been the subject of intense investigation. Efforts to track down postnatal TEPC have focused on determining the potency of TEC subpopulations in the adult mouse thymus, using the combination of in vitro and in vivo assays. Perhaps

the most salient question in this regard is whether a multipotent stem/progenitor cell exists that can maintain the highly heterogenous TEC population or if distinct compartments are exclusively sustained by precursors that are lineage restricted. This question is particularly important with respect to strategies that aim to reconstitute thymus function.

The existence of an mTEC-restricted progenitor cell in the adult mouse thymus has been suggested by both transplantation-based assay and lineage tracing studies [181,184,185]. In particular, Sekai et al. identified a rare mTEC population in the postnatal mouse thymus that exhibited a similar phenotype to fetal mTEC restricted precursors (SSEA-1^+CLDN3,4^{hi}, $MHCII^-UEA\text{-}1^-$). Purified populations of these cells could contribute to mature mTECs within transplanted reaggregate fetal thymic organ cultures (RFTOCs) based on E14.5 WT thymi, although this capacity was significantly reduced compared to cells isolated during embryonic and neonatal stages [181]. Importantly, contribution to the cTEC lineage was not detected. Interestingly, the authors found that this decline in progenitor mTEC activity was associated with the presence of thymocytes at advanced differentiation stages, as SSEA-1^+ cells isolated from $RAG2^{-/-}$ mice, in which later stages of thymocyte development are blocked, retained a higher capacity for mTEC reconstitution. The mechanistic reason for this is unclear, although it may reflect the developmental arrest of mTEC precursors as a consequence of impaired formation of the $RAG2^{-/-}$ thymic medulla.

Evidence of bipotent TEPCs in the adult thymus has also recently emerged. One study has identified an $EPCAM^+UEA\text{-}1^-MHCII^{lo}Ly51^{lo}$ population that resides at the CMJ and can contribute to both cortical and medullary lineages upon transplantation [310]. However, these experiments used high input cell numbers (7.5×10^4–1×10^5), limiting the utility of the assay with respect to determining lineage contribution from single cells. A more recent study identified an $EPCAM^+PLET1^+MHCII^{hi}Ly51^+UEA\text{-}1^-$ population, also residing at the CMJ in adult thymus, that could contribute to both cTEC and mTEC lineages, a capacity that persisted for at least 9 months [110]. Importantly, this ability was evident with input cell numbers as low as 129/graft, with limiting dilution analysis predicting the frequency of bipotent cells within the population at around 1:90. Furthermore, the long-term contribution exhibited by this population points to a possible stem cell activity. Interestingly, this study also found cTEC-restricted lineage potential in a cell type that overlaps phenotypically with the $EPCAM^+UEA\text{-}1^-MHCII^{lo}Ly51^{lo}$ population [310], providing a possible explanation for the multilineage contribution observed for the $MHCII^{lo}Ly51^{lo}UEA\text{-}1^-$ compartment [110].

A thymic epithelial stem cell (TESC) population with multilineage potential has also been reported [311]. This study assayed the ability of adult thymic stromal cells to develop in vitro spheroids, an approach that has been employed for other self-renewing epithelia such as the mammary gland. The authors found that the spheroids, termed "thymospheres," were clonally derived, displayed some degree of self-renewal and exhibited multilineage potential [311]. However, the main conclusions have been refuted in a recent study that showed that $FOXN1^-$ thymic mesenchymal cells initiate thymosphere formation by recruiting $FOXN1^+$ TECs [312], providing insight into the role of nonepithelial thymic stroma in supporting thymus development and function.

Strategies for thymus reconstitution

Reconstitution of thymus function is essential for strategies that aim to restore immune function in a number of clinical settings. In particular, optimal regeneration of a functional T cell repertoire following BM transplantation depends on robust thymus function, which is impaired in many patients as a consequence of thymic involution. Several approaches may be considered to achieve this end. In vitro generation of a functional thymus is one strategy, which could be employed to either facilitate the production of a T cell repertoire for adoptive transfer or to provide a source of engineered tissue for transplantation. An alternative approach is to restore endogenous thymus function by reversing thymic involution. In the former, a scalable source of cells that can generate the full range of functional TEC is essential, while for the latter, an intricate knowledge of how TEC maintenance and function is regulated at the molecular level is required. The advances in our understanding of thymus biology discussed in this review have led to significant developments in each of these areas, making the goal of thymus reconstitution a tangible reality.

One possible source of TEC are pluripotent stem cells, which have the capacity to generate all cell lineages that constitute functional tissues and organs. This approach can utilize either ES cell lines derived from blastocysts or induced pluripotent stem cells (iPS) generated by reprogramming somatic cells. The use of iPS cells has the key advantage of enabling the generation of cells that are genetically identical to individual patients, thus circumventing the need for immunosuppression. The viability of this approach depends on the ability to efficiently direct the differentiation of PSCs to the TEC lineage. In this regard, several studies have now reported progress toward generating TEC-like cells derived from in vitro cultures of PSCs [206–210]. Notably, two studies have used in vitro protocols that promote the stepwise differentiation of human ESC lines into definitive endoderm, foregut endoderm, pharyngeal endoderm and subsequently epithelial cells bearing hallmarks of TEPCs, including *Foxn1*

expression [209,210]. When grafted into athymic nude mice, these TEPC-like cells were able to differentiate into cells that expressed markers associated with cTECs and mTECs, as well as support some level of thymopoiesis. However, the grafts derived from TEPC like cells did not display the tissue architecture typical of the functionally mature thymus, with no distinct cortical or medullary regions apparent. This is important as the spatial organization of the TE is critical to ensure that T cell−development proceeds through the correct sequence of events. Furthermore, the induction of the distinct cortical and medullary architecture itself depends on reciprocal interactions with developing thymocytes that have transitioned through the various stages of maturation. The highly ordered spatial organization of TECs is therefore indicative of functional thymopoiesis, and the lack of an organized TE in grafts derived from TEPC-like cells suggests that thymus function has not been fully recapitulated and/or sustained. Thus while this avenue of research is promising, further progress is needed to establish if TEPC-like cells derived by these methods can fully reconstitute thymus function.

Recently, two studies have revealed that the manipulation of FOXN1 expression presents a powerful means of both restoring thymus function in the aged thymus [280] and directly reprogramming cells into functional TEC [283]. In the former, induction of FOXN1 expression in TECs of aged mice was sufficient to reverse thymic involution and restore cortical and medullary organization to close to that observed in the young, preinvolution thymus. Consistent with this, thymopoiesis was restored, demonstrated by the presence of thymocyte subsets and newly generated peripheral T cells. Strikingly, this effect could be elicited in mice as old as 2 years. The significance of this study is it reveals that targeting a single transcription factor is sufficient to reverse thymic involution and validates FOXN1 as a target for thymus regeneration, further highlighting the importance of understanding how this transcription factor itself is regulated.

In the second study, FOXN1 expression was induced in in vitro cultures of murine embryonic fibroblasts [283]. This resulted in the generation of cells with TEC-like characteristics, including the expression of genes required in TEC to promote T cell commitment and development, such as DLL4, CCL25, and KITL, as well as the endogenous FOXN1 locus. Consistent with this, these induced TEC (iTEC) could support in vitro thymocyte development in a similar manner to the OP9-DLL4 system. Strikingly, when iTECs were grafted into athymic nude mice, they could generate a fully functional thymus that supported thymopoiesis and the generation of peripheral T cells. Critically, thymus tissue derived from iTECs displayed the cortical and medullary architecture typical of the functionally mature gland, and the phenotypic heterogeneity expected of fully mature cTEC and mTEC. The generation of iTEC by forced expression of FOXN1 identifies for the first time a means of directly reprogramming cells to the TEC lineage. This represents a strategy that could enable the generation of transplantable thymus tissue from a highly scalable source of cells and thus is highly significant with respect to restoring immune function. In this regard it will be important to determine which cell types are competent to form iTEC in response to forced FOXN1 expression.

Collectively, our understanding of the cellular and molecular mechanisms that regulate TEC development and maintenance have led to significant progress in developing strategies that will enable the reconstitution of thymus function. Continued advances in our knowledge of thymus biology will accelerate this progress toward therapeutic strategies that can be utilized in the clinic.

Summary

Thymus organogenesis is a complex process in which a dynamically regulated, three-dimensional organ forms from the endoderm of the 3PP. The TE, a critical regulator of thymopoiesis, is comprised of many subtypes of TEC, all of which arise from a common progenitor. Importantly, T cell development depends on interactions with both cortical and medullary TEC subtypes, which mediate different aspects of T cell differentiation and repertoire selection. Therefore the development of functional in vitro or transplantable thymic organoids requires the ability to recapitulate all of these functions, with appropriate spatial and temporal constraints. Recent studies have begun to clarify the lineage relationships of the different TEC subtypes, and to unravel the molecular networks that govern thymus and parathyroid organogenesis. However, with respect to the goal of developing in vitro or transplantable thymi for therapeutic purposes, several significant questions remain unresolved:

- What factor or factors specify thymus and parathyroid lineages within the 3PP?
- How does the network of transcription factors and signaling molecules that are expressed in the endoderm and surrounding mesenchyme/mesoderm control subsequent thymus and parathyroid development, including the maintenance, proliferation, and differentiation of thymic epithelial and parathyroid progenitor cells?
- How is *Foxn1* regulated in fetal and adult TEC?

In addition, further identification of the transcriptional networks in which FOXN1 operates to control development and function of c- and mTEC, is an important goal. The potential of factors downstream of FOXN1 to facilitate engineering of HPC niches ex vivo has already been demonstrated by transgenic expression of varying

combinations of *Ccl25*, *Cxcl12*, *Scf*, and *Dll4* in *Foxn1*-deficient TE [313]. It is likely that the knowledge of both additional FOXN1-regulated genes and proteins required in conjunction with FOXN1 to regulate specific functions in TEC may prove useful for manipulating or engineering thymus function(s) in vitro or in vivo.

Finally, a deeper understanding of the mechanisms that mediate the homeostatic maintenance of the mature thymus is critical for developing the strategies for thymus regeneration or replacement. Of particular importance is continued delineation of both the stem/progenitor cell types responsible for replenishing TEC in the adult thymus, and of the mechanisms that operate on TEC to induce thymic involution. Progress in each of these issues is likely to facilitate rational design of strategies for therapeutic reconstitution of the adaptive immune system. The development of such strategies should significantly impact the health of the aging population, and other immunocompromised individuals.

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Part Eleven

Gastrointestinal system

Chapter 38

Stem and progenitor cells of the gastrointestinal tract: applications for tissue engineering the intestine

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Introduction

The intestinal epithelium performs multiple complex functions, including absorption of nutrients and providing a barrier against pathogens. These epithelial cells are exposed to a harsh luminal environment, necessitating constant self-renewal and replenishment of the majority of the epithelium every 4−8 days. The intestinal stem cell (ISC) long remained elusive, but over the past few decades considerable advances have been made to identify progenitor populations and develop a more cohesive understanding of intestinal renewal.

Stem cells are, by definition, capable of both self-renewal and differentiation, with enormous potential for future therapies. The identification of different stem cell populations in the crypts, and insight into the complex network of signaling molecules that sustain them, has led to the advent of techniques that take advantage of these properties. Tissue engineering may capitalize on native regenerative populations or strategies to recapitulate aspects of development. Following the identification of native stem/progenitor populations, tissue-engineered intestine (TEI) has been produced from multiple sources. TEI might have the potential to restore enteral independence in patients with short bowel syndrome (SBS), a devastating condition that results from extensive loss of the absorptive capacity of the epithelium, resulting in partial or total dependence on intravenous, or parenteral, nutrition for survival. Here we will discuss the stem/progenitor cells of the gastrointestinal tract, chemical messengers that maintain the stem cell niche, and future applications for tissue engineering that might apply these concepts in order to develop future human therapies.

Stem cells of the intestine

Cell types of the epithelial layer

The intestinal epithelial layer is the most rapidly cycling tissue in mammals and is structurally adapted to provide maximum absorptive surface area. The wall of the intestine is composed of four layers, the innermost mucosa, submucosa, muscular layer, and outer serosa (Fig. 38.1). The microscopic anatomy consists of the invaginated crypts of Lieberkühn surrounding the finger-like villus projections (Fig. 38.2A). Intestinal epithelial cells can be divided into two distinct lineages: absorptive and secretory (Fig. 38.2A). The absorptive cells or enterocytes function in uptake of nutrients and are localized to the villi. There are several types of secretory cells which aid in protecting and optimizing the function of this absorptive surface. The goblet cells secrete mucus that helps one to protect the enterocytes and serves as a passive barrier

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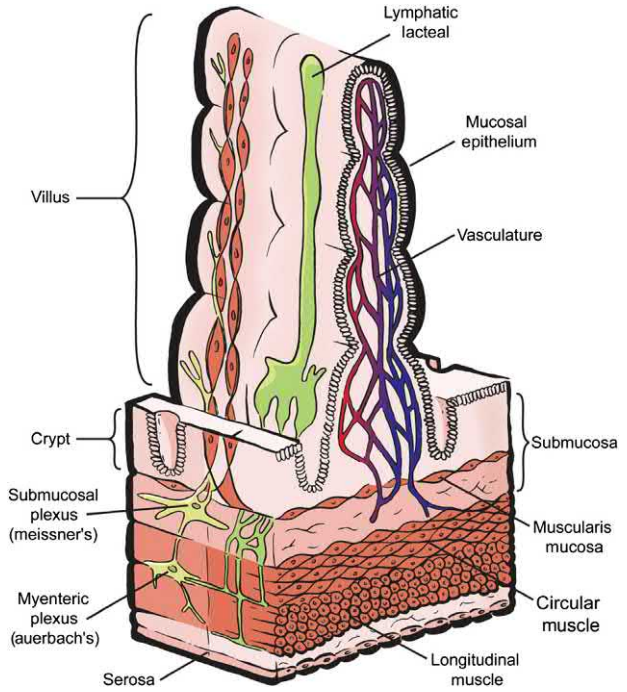

FIGURE 38.1 Structure of the small intestinal villus. A cross section of an individual villus and surrounding crypts depicts the vasculature, lymphatic, and neuromuscular structures. Small intestinal villi are finger-like projections covered in absorptive enterocytes with dense microvilli that provide an extensive absorptive area for extracting nutrition from the gastrointestinal lumen. Arterial vessels feeding the muscular and mucosal layers originate from the arterial plexus of the submucosa, with the mucosal layer receiving roughly 80% of the intramural blood flow. Submucosal arterioles are eccentrically located within the villi, which pass to the villus tip forming a capillary network with multiple anastomoses and egress as a single eccentrically located venule. The lymphatic system of the small intestinal villus comprises a centrally located lacteal with the apical portion containing endothelium that provides propulsion of absorbed luminal contents toward the collecting lymphatics when the villus contracts. The neuromuscular interface not only promotes contractility from ganglia within the myenteric and submucosal plexuses within the villus, but enteric neural connections with neuropods of enteroendocrine cells establish a neuroepithelial networks allowing propagation of luminal signaling to the nervous system.

against pathogens [1]. M cells overlie Peyer's patches and serve as antigen sampling cells that aid in initiating the adaptive immune response and act as an active barrier to infection [2]. Enteroendocrine cells produce and secrete numerous hormones involved in regulating a wide range of physiologic processes, including intestinal motility and metabolism [3]. The function of tuft cells has not been well elucidated, but among other possible purposes, they appear to function as chemosensors [4,5] and local sources of prostaglandins [6]. Finally, Paneth cells are found within the crypts between villi, secrete antimicrobial peptides, and provide essential Wnt and epidermal growth factor (EGF) ligands for the crypt base progenitor stem cells [7].

Stem and progenitor cell types

Due to continuous exposure to harsh luminal conditions, routine turnover and self-regeneration are important for the maintenance of the epithelial barrier. Intestinal homeostasis is a delicate balance between proliferation within the crypts and shedding of mature cells from the upper third of the villi. Several foundational lineage-tracing studies have localized intestinal progenitor or stem cells (ISCs) to the base of the crypts, with daughter cells differentiating and migrating out of the crypt. Cell expansion and the beginning of differentiation occur within the so-called transit amplifying (TA) zone beginning at the +5 region, or five cells above the base of the crypt (Fig. 38.2B and C). With the exception of Paneth cells (see later), cells differentiate as they move out of this region and up the villi until they are shed at the end of their life cycle [8–11]. The intestinal progenitor/stem cells located within the crypt are drivers of cell repopulation through proliferation.

Historically, there have been two competing models of ISC identity: "the stem cell zone model" and the "+4 model" [12]. The "stem cell zone" model was first postulated by Cheng and Leblond who noted wedge-shaped crypt base columnar (CBC) cells interspersed between Paneth cells at the crypt base [13,14]. Lineage labeling, performed by tracking phagocytosed debris from injury caused by tritiated thymidine injection, demonstrated that these cells could give rise to other, differentiated, cell types [14]. Further work, including mutagenesis lineage tracing, revealed both long- and short-lived cells within the crypt; the long-lived cells, thought to be the multipotent CBC cells, gave rise to various daughter cells. The central premise of this model is that the CBC cells reside in the stem-cell permissive crypt and the daughter cells migrate to the +5 position where local signals induce lineage commitment and differentiation [15–17]. The CBC cells represent a rapidly cycling ISC population that can be identified morphologically based on their unique shape, biochemically based on molecular markers (see below), and functionally by their persistence in an undifferentiated self-renewal state with the ability to produce all mature intestinal epithelial cell types [8].

The first widely accepted ISC marker, leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), marks the CBC cells. Studies using mouse models and organoid cultures showed that Lgr5+ cells have the essential characteristics of stem cells, including the ability to self-renew and persist, rapidly cycle, and to give rise to all mature intestinal cell types [18,19]. Lgr5 is a G-protein coupled receptor that binds R-spondin and serves as a Wnt signaling amplifier [20,21]. Lgr5+ ISCs also express achaete-scute family bHLH transcription factor 2 (ASCL2). *Ascl2* knockout studies performed in mice

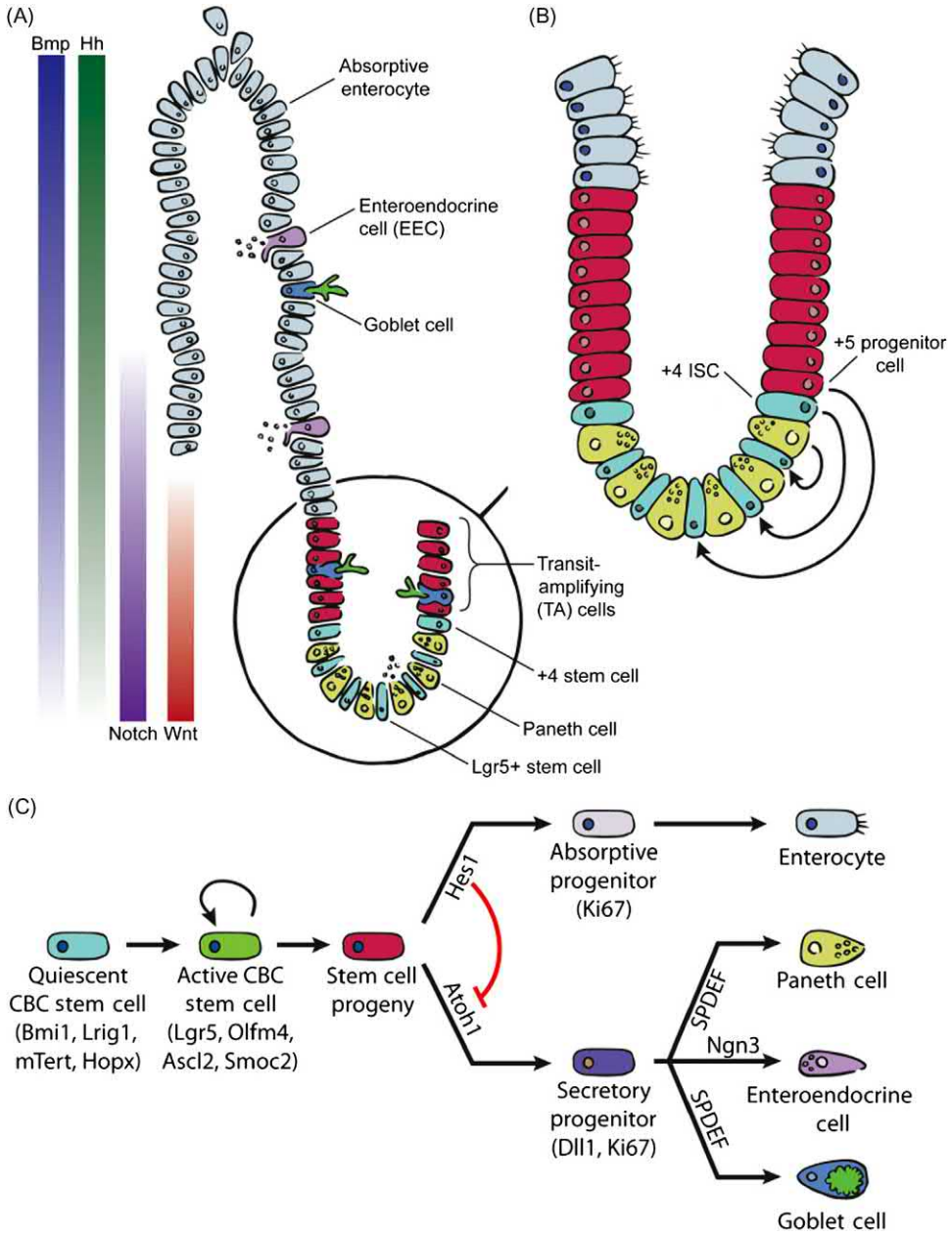

FIGURE 38.2 **Small intestinal epithelium and the stem cell niche.** (A) The villus and crypt contain the epithelial cell types necessary for mature function and self-renewal of the small intestine. The villus contains absorptive and secretory enterocytes. The secretory cell−type goblet cells (blue), enteroendocrine cells (purple), Paneth cells (yellow) are located within the crypt base. Maintenance and activation of the ISC pool involves a complicated interplay of signaling between pericryptal stromal and adjacent epithelial cells. A relatively high expression of Bmp, Hedgehog (Hh) restrict the stem cell population to the base of the crypts. Within the TA zone and crypt, high levels of Notch and Wnt maintain stemness and promote proliferation of ISCs within the crypt. (B) The ISC compartment of the small intestine contains CBC cells interspersed among protective Paneth cells in the active stem cell zone. The quiescent label-retaining ISC resides near the bottom of the crypt in the +4 position (quiescent stem cell zone) just above the interspersed Paneth cells. Acute injury results in the loss of the proliferating Lgr5+ stem cells but preserves the relatively resistant Paneth cell precursors, +4 stem cells, and niche cells. When ISCs are damaged or die, neighboring +4 ISC can repopulate CBC within the crypt base. Alternatively, Paneth cells and absorptive/secretory progenitors within the TA zone can undergo dedifferentiation in order to restore the ISC population within the crypt. This remarkable plasticity allows for survival and regenerative capacity of intestinal epithelium even under the harshest of conditions. (C) Identification of distinct and overlapping ISC markers has relied on intricate lineage-tracing studies. Active-cycling ISC (green) and quiescent ISC (cyan) express distinct stem cell markers, although some overlaps have been demonstrated for Bmi1, Hopx, mTert, and Lrig1. The +5 progenitor cell undergoes cell lineage fate decisions through expression of Atoh1 or Hes1. Absorptive progenitor cells arise from Hes1 expression, which has an inhibitory effect on Atoh1 and subsequent production of secretory enterocytes. Although many of the factors responsible for specific epithelial cell differentiation have yet to be elucidated, SPDEF and Ngn3 (neurogenin 3) promote the formation of selected secretory enterocytes. *CBC*, Crypt base columnar; *ISC*, intestinal stem cell; *TA*, transit amplifying.

identified a concomitant loss in Lgr5 + CBC cells, suggesting that Ascl2 is essential for stem cell survival or identity [22]. In addition, Ascl2 has been recently identified as a transcriptional switch that is regulated in a direct Wnt-responsive loop, driving genes crucial to maintaining the stem cell state [23].

In contrast, the "+4" ISC model was proposed by Potten after identifying radiosensitive, label-retaining cells (LRCs) located immediately above the Paneth cell position, usually four cells above the lowest point in the crypt [12]. Theoretically, ISCs that were LRCs could retain label through asymmetrical inheritance of chromosomes, retaining an "immortal strand" and preventing radiation-induced damage from exerting a lasting effect on the genome [24–26]. However, asymmetrical division of ISCs is highly controversial [27] and does not appear to occur in the rapidly cycling CBC cells [28]. The +4 cells are now thought to represent a reserve population of quiescent stem cells as opposed to the rapidly cycling CBC cells which provide the major source for homeostatic cell turn over [12,14,29]. Interconversion between these populations has been an evolving puzzle, with Lgr5 + cells shown to produce +4 cells and cells in the +4 position producing CBC cells in special conditions [30,31].

Lineage-tracing studies initially identified *Bmi1*, *Hopx*, *Lrig1*, and *mTert* as potential markers distinguishing the slowly cycling +4 ISCs from the rapidly cycling CBCs [32–35]. The Capecchi lab found *Bmi1* expressed discretely in the +4 position, and like LGR5 + cells, +4 cells differentiated into all of the epithelial cell types [33]. Bmi1 + cells have been shown to revert to Lgr5 + ISCs after ablation of the Lgr5 + CBCs and have been posited to play a role in epithelial regeneration after radiation damage [36,37]. However, these markers have not been definitively validated as further studies have found that *mTert* and *Bmi1* are expressed throughout the crypt, while *Hopx* and *Lrig1* expression is actually highest in Lgr5 + cells [38,39].

In addition to the two "traditional" ISC populations, recent evidence shows that much of the epithelium can undergo reversion to an Lgr5 + stem phenotype when necessary. For example, alkaline phosphatase-expressing cells (absorptive enterocyte lineage) can dedifferentiate and become Lgr5 + ISCs [40]. Secretory progenitors and enteroendocrine cells can revert after injury, as can Paneth cells [40–43]. A role for Paneth cells in replenishing lost ISCs is particularly interesting given the unique nature of this population; while most cells migrate up out of the TA zone as they differentiate, Paneth cells migrate down to the bottom of the crypt where they are intercalated with CBC cells. They are longer lived than other terminally differentiated intestinal epithelial cells, with a turnover time of approximately 57 days [44]. They provide factors that maintain the stem cell niche, particularly Wnt, and organoid cultures with coplated Lgr5 + cells and Paneth cells have greatly increased growth efficiency [7]. Inhibition of glycolysis in Paneth cells impairs organoid growth, indicating that they may also provide metabolic substrates such as lactate to Lgr5 + stem cells [45]. Furthermore, while they are able to dedifferentiate and regain multipotency, they only seem to be competent to do this after an injury [46]. Together, these studies and others show that the stem cell niche is a complex environment sustained in part by interconversion of cell types. A deeper understanding of the cues that can drive this cell plasticity could lead to translationally relevant approaches for improving tissue-engineered small intestine (TESI) yields.

Signaling pathways in the intestinal epithelium

The complex homeostatic mechanisms of the stem cell niche, which maintain the balance between proliferation and differentiation, have yet to be fully elucidated. While epithelial–epithelial interactions play a vital role in gut homeostasis, there is also significant crosstalk between the mesenchyme and epithelium [47,48]. ISCs are essential to the intestine's response to injury, and while reserve ISC populations appear to be integral to this process, the exact mechanism by which this occurs is poorly understood [32,36,43]. For reviews of intestinal development, see Chin et al. [49] and Le Guen et al. [47]. Here, we focus on a few of the known signaling pathways that control the stem cell niche and contribute to growth and differentiation, particularly Wnt, Notch, ErbBs, Hedgehog, and BMP.

The Wnt pathway

Wnt signaling is a critical stimulus for stem cell proliferation. There are two primary ways the Wnt pathway can be activated: via a β-catenin/T-cell factor (TCF)–dependent mechanism (the canonical pathway) and a β-catenin/TCF-independent mechanism (the noncanonical pathway) which is reviewed elsewhere [50,51]. The canonical pathway is required for maintenance of the proliferative zone and Lgr5 + ISCs in the crypt [52,53] and regulates Paneth cell differentiation [54]. Wnt ligands bind to the Frizzled receptor and its coreceptor LRP5/6 in order to inhibit the formation of the Axin, APC, CKI, and GSK3β complex that usually degrades β-catenin [48,55]. The inhibition of this routine destruction allows β-catenin to accumulate and travel to the nucleus, forming a complex with heterodimer lymphoid enhancer factor and TCF. This complex of transcription factors activates the expression of Wnt target genes, including Axin2, cMyc, CD44, Ccnd1, Sox9, Lgr4/5, and Cdx1, among others [55,56],

which are involved in driving cell proliferation and self-renewal.

Constitutive activation of the Wnt pathway via APC mutation results in polyposis and epithelial hyperproliferation [48,56]. On the other hand, a loss of Wnt function results in the absence of intestinal crypts and the sole presence of differentiated epithelial cells. Pharmacological deletion of Porcn, a protein important in the palmitoylation and proper secretion of Wnt for paracrine signaling, results in a decrease in Lgr5 + ISCs and the loss of the Wnt-induced marker of OLFM4 + CBC cells [55]. Looking downstream from Wnt, the loss of Wnt effectors and target genes alters crypt formation, size, and proliferation in adulthood [55,57]. Wnt is also an important mediator of other signaling pathways, including Eph/Ephrin, Notch, and BMP [48].

Wnt signaling components are expressed, possibly redundantly, within the epithelium and the mesenchyme; for example, both Paneth cells and subepithelial telocytes express Wnt ligands [58,59]. Wnt activity thus exists in a gradient along the crypt-villus axis, with the strongest signal at the crypt base. Paneth cells produce Wnt3 that has been shown to establish a short-range gradient within the crypt, and the presence of Paneth cells augments the growth of organoids [7,60]. However, single Lgr5 + cells have been observed to form organoids without Wnt supplementation, albeit with very low efficiency, and demonstrate growth arrest with Wnt inhibition [7,61]. Several in vivo studies have shown that development and homeostatic turnover can proceed without *Wnt3* or Paneth cells [59,62,63], though in the absence of this support the ISCs respond poorly to injury, demonstrating a critical role for Paneth cells and their products in maintaining the stem cell niche in the face of a challenge [64].

The Notch pathway

Notch proteins represent a highly conserved set of transmembrane signaling molecules that aid in cell−cell communication and cell differentiation. In the intestine, Notch regulates crypt cell proliferation while maintaining the balance of secretory and absorptive cells. Notch activity requires cell−cell interaction and specifies cell fate in adjacent cells through lateral inhibition [65,66]. Binding Notch ligand Jagged or Delta-like (Dll1/4) causes the Notch receptor on adjacent cells to be proteolytically cleaved. ADAM10 is responsible for cleaving the extracellular portion, while γ-secretase releases an intracellular fragment, termed the Notch intracellular domain (NICD). NICD travels to the nucleus and, along with cofactor RBPJK, induces expression of *Hes1/5/7*, *Hey1/2*, and other targets [65,67]. In cells receiving Notch signaling, the expression of Notch ligands Dll1/4 and Jagged is inhibited by Notch target genes. Therefore Notch-receiving cells cannot themselves transmit the Notch signal.

In the crypt base the combined presence of Notch and Wnt signals maintains stemness [56,65]. Traveling up the crypt, decreased exposure to Wnt and Notch, results in an absorptive fate in differentiated cells [68]. In contrast, Wnt signaling in the absence of Notch results in development toward secretory fate. In the absence of Wnt, the presence/absence of Notch signaling is sufficient to determine absorptive/secretory fate of intestinal cells [69]. Indeed, the expression of Notch target *Hes1* represents a precursor of absorptive fate in the intestine [65].

Epidermal growth factor receptor/ ErbB signaling

EGF receptor (EGFR/ErbB1) is the prototypic member of a family of four receptor tyrosine kinases that also includes ErbB2/HER2, ErbB3, and ErbB4 [70]. These receptors recognize and are activated by, with varying specificity and affinity, a broad panel of ligands, including EGF, heparin-binding-EGF-like growth factor, epiregulin, betacellulin, amphiregulin, transforming growth factor-alpha, and the neuregulin/heregulin peptides. Ligand binding stabilizes receptor homo- and/or heterodimers, resulting in increased tyrosine kinase activity and phosphorylation on c-terminal tyrosines that in turn provide docking sites for downstream substrates and signaling partners [70,71]. ErbBs can activate a wide array of signaling pathways, such as Ras/Raf/MEK/ERK, PI3K-Akt, JNK, JAK-Stat, Src, Rho/Rac, and PLC-γ/PKC [72,73], with bias in the downstream pathways providing part of the selectivity in response [74,75]. Specificity in responses is likely driven by ligand−receptor affinity together with the balance of available ligands and receptors expressed in a cell.

ErbB signaling supports the maintenance of stem/progenitor cells in the gut. Ligand-secreting mesenchymal cells reside along the bases of the intestinal crypts, and Paneth cells secrete EGF. Isolated ISCs require exogenous EGF to efficiently survive and expand ex vivo [76], underscoring the importance of EGFR/ErbB1. Deletion of another family member, ErbB4, compromises the ISC niche and Paneth cell compartment in expanding enteroid cultures [77,78]. In addition, ErbBs strongly activate the PI3K-Akt pathway, and Akt signaling can lead to activation of the canonical Wnt pathway by way of β-catenin phosphorylation [79]. Prolonged activation of PI3K-Akt by PTEN deletion promotes ISC activity, crypt fission, and intestinal polyp formation [80]. Taken together, the literature supports key roles for ErbB receptors in the proliferation and survival of ISCs.

The Hedgehog pathway

The Hedgehog (Hh) pathway drives an epithelial−mesenchymal signaling loop that decreases epithelial proliferation in the crypt [56,66]. Vertebrate Hedgehog genes, including *Indian hedgehog* (*Ihh*), *Sonic hedgehog* (*Shh*), and *Desert hedgehog* (*Dhh*), are expressed in the intestinal epithelium and signal the mesenchyme in a paracrine fashion [48,81]. Hh precursors are cleaved and lipid modified in Hh-expressing cells to be released through the activity of dispatched (Disp). Hh molecules then bind to the Patched receptor (Ptch1/2) and coreceptors Cdo, Boc, and Gas1 resulting in a relief of inhibition of Smo. This induces a signal cascade resulting in the dissociation of Gli1/2/3, which are the transcriptional inducers of the Hedgehog pathway. Hh targets include *Ptch1/2*, *Gli1*, *Cyclin D*, and *Hhip*, among others [81−84].

Shh in the adult intestine is most highly expressed in the crypt, where it marks undifferentiated epithelial cells [48,67]. Hh signaling is required for orderly formation of villi and for regulation of crypt density. Overexpression of *Hhip*, a target of Hh signaling and also a Hh inhibitor, results in a lack of villi, crypt hyperplasia, and incomplete epithelial differentiation [48,81]. A decrease in Ihh results in increased wound healing responses due to intestinal epithelial proliferation and crypt fissioning [85]. Hh signaling is thus important in the maintenance of the mesenchymal and epithelial homeostasis.

The BMP pathway

BMPs are part of the TGF-β family of signaling molecules and targets of both Wnt and Hh signaling [48,56]. BMP activation results in the phosphorylation of SMAD 1/4/5/8, which then form a heterodimer (SMAD4 with SMAD 1, 5, or 8) that travels to the nucleus to induce the transcription of target genes [47,48,67]. BMP ligands are primarily expressed in the mesenchyme, where Hh signaling from the epithelium induces their expression; conversely, the primary activity of BMP ligands expressed by the mesenchyme is to signal through receptors located in the epithelium [48].

BMPs are expressed most strongly in the intervillus mesenchyme, where their localized activity is restricted by the concomitant expression of BMP inhibitor Noggin at the +4 position, just above the intestinal crypt proper. Ectopic cryptogenesis is seen when intestinal BMP is inhibited in a mouse model, a finding likely associated with a relief of inhibition of the canonical Wnt pathway [86,87]. Both of these signals are important in the maintenance of ISCs, and their crosstalk and patterning suggest a role for BMP expression in normal villification/cryptogenesis [88]. Indeed, studies show that molecules, including BMP4, cluster beneath developing villus tips that act to restrict ISCs to the developing crypt region [88,89].

Tissue engineering the intestine with stem/progenitor cells

With the identification of the ISC and associated growth factors, manipulation of ISC to generate TEI has become possible. Many tissue-engineering approaches include delivering various stem/progenitor cells on synthetic or biological scaffolds in vivo with the long-term aim to reproduce a construct that is similar in structure and function to native intestine that could eventually replace enough lost intestinal digestive/absorptive surface area to reduce the complications from SBS and its therapies (Fig. 38.3).

We apply the term TESI to tissues that recapitulate all of the key components of the intestine and have proposed various other terminology to reflect the regions of the gastrointestinal tract that can also be produced, such as tissue-engineered esophagus (TEE), tissue-engineered liver (TELi), tissue-engineered stomach (TES), and tissue-engineered colon (TEC) [90−100]. The overall concept of TEI, which may be from any region, is commonly termed TEI. These terms ought not to be applied to biologic constructs that lack function (although physiologic function may not be exactly replicated) and do not support the key cell populations required for function, such as systems, in which epithelium or mesenchyme survives alone. We distinguish these TEI variations versus disease in a dish or organ-on-a-chip systems, which may be informative, but lack important components of the desired gastrointestinal tissue as well as systemic influences. Macroscopically, in order to eventually be a long-term solution for patients with SBS, TESI must recapitulate native intestinal epithelial mucosa, submucosa, smooth muscle, and serosa in the correct orientation. Intestinal motility, digestion, absorption, secretion, and excretion also require reconstitution of an enteric nervous system (ENS) which is not derived from ISCs but from the vagal neural crest [101]. In addition, an adequate vascular and lymphatic supply is required for oxygen, nutrient, and waste exchange [102]. On a subcellular level, appropriate barrier function, digestion, and absorption depend on the correct localization of enzymes and membrane proteins [103].

Organ-specific stem cell progenitors versus pluripotent stem cells

Pluripotent stem cells, multipotent progenitor cells, terminally differentiated cells, or a combination of these have been investigated for the generation of TEI

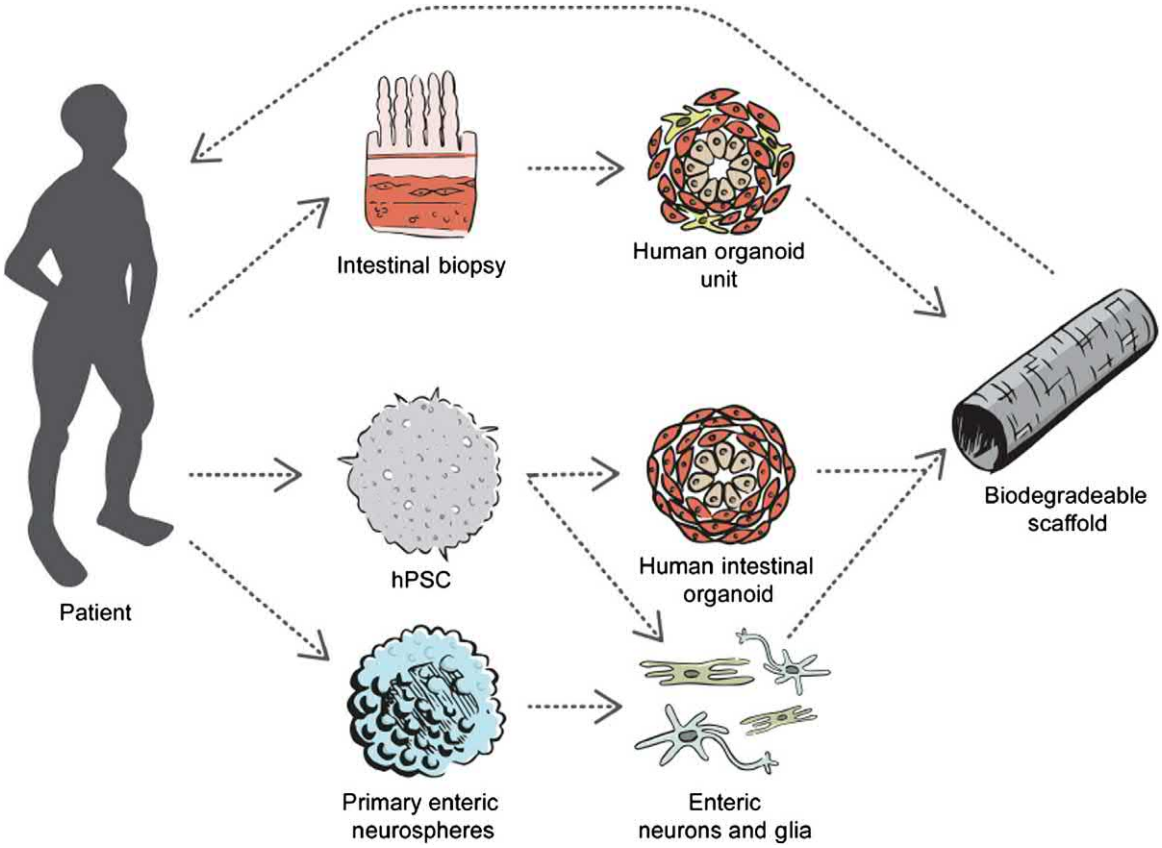

FIGURE 38.3 Methods for creating tissue-engineered small intestine. TESI can be created via different approaches. Primary intestinal biopsies obtained through minimally invasive endoscopic approaches could be mechanically dissected and enzymatically dissociated to form epithelial/mesenchymal cell clusters termed hOU. hOU contain all of the necessary cell types required for intestinal absorption and mechanoluminal function, including mature epithelial cell types, myofibroblasts, and cell types of the ENS. Small intestinal hOU can be cultured or directly placed onto biodegradable scaffolds that, once implanted into the omentum, mesentery or other highly vascularized intraabdominal sites, can produce TESI that can be anastomosed to native intestine. Another recent approach is to generate TESI derived from patient-specific iPSCs (hiPSC) or human leukocyte antigen-matched human embryonic or iPSC banks to generate TESI. With directed differentiation methods, hPSC can be directed in culture to form HIO that contains many of the necessary cell types of the intestine. However, in contrast to primary intestinal-derived OU, HIO fails to generate ENS cell types. Therefore in order to restore function to these constructs, ENCC derived from hPSC or enteric neurosphere extraction from primary intestinal biopsies must be added back to developing HIO-derived TESI. A combination of HIO and ENCC derived from hPSC or enteric neurospheres loaded onto biodegradeable scaffolds can be implanted into the omentum, mesentery, or other highly vascularized locations within the patient to allow for ENCC-HIO-TESI to grow and later be connected to native intestine. The benefit of these approaches is that they are amenable to genetic manipulation prior to implantation. With the advent of highly specific targeted genetic manipulation through CRISPR/Cas9, ZFNs and TALENS, OU, attributes important to intestinal absorption may be enhanced. This approach may become a crucial addition to enhancing the function of tissue-engineered organs. *ENCC*, Enteric neural crest cells; *ENS*, enteric nervous system; *HIO*, human intestinal organoids; *hOU*, human organoid units; *iPSCs*, induced pluripotent stem cells; *OU*, organoid units; *TESI*, tissue-engineered small intestine, *hiPSC*, human induced pluriopotent stem cells.

[94,104–108]. Much of the initial works performed in initial experiments employed organoid units (OU), which are made by mechanically and chemically digesting native intestine [109]. They are distinct from organoids or enteroids in that they contain both epithelial and mesenchymal elements. Yet other approaches focus on implanting cultured enteroids, clusters of intestinal-derived epithelial cells, that were genetically altered to enhance digestive and absorptive function. Pluripotent stem cells, either induced pluripotent stem cells (iPSCs) or embryonic stem cells, can be differentiated into all of the layers of the small intestine, excepting the ENS, and these are termed human intestinal organoids (HIO) [110].

Synthetic and biological scaffolds

The most common approach to tissue engineering small intestine starts with creating a structural template with a scaffold for cellular growth. The scaffold is a biological or synthetic biodegradable matrix that must maintain its shape, provide an appropriate surface for tissue growth and organization, and be porous enough to allow for

angiogenesis and vasculogenesis [111,112]. Scaffolds provide three-dimensional mechanical support for cells that recapitulate the microcellular environment thereby guiding reorganization into functional tissues. Scaffolds can be designed from several synthetic polymers or extracted from primary tissues through decellularization of native organs [111,112].

We have routinely noted poor tissue growth in decellularized tissues as well as in other relatively nonporous polymers and have demonstrated that high porosity is associated with improved stem/progenitor cell growth [99]. Further modifications can provide additional signaling factors or extracellular matrices, such as collagen, laminin, heparin sulfate, and others, that may influence cellular proliferation, differentiation, and migration [112,113]. The scaffold commonly employed in our laboratory is polyglycolic acid/poly-L-lactic acid (PGA/PLLA), a synthetic fiber that can be woven or felted into porous sheets and coated with extracellular matrices and growth factors which permit initial nutrient support and oxygen diffusion required for cell survival prior to neovascularization [111,112]. Engineered tissues have been derived from small intestine, colon, esophagus, stomach, and liver by delivering cells on this material and the generation of TESI will be described in more detail later [90,93–97,100,104]. As cells proliferate, secrete extracellular matrix, and establish appropriate organization through cell-to-cell interactions within the developing engineered tissue, the supportive scaffold is gradually degraded by hydrolysis.

Primary intestinal-derived organoid units

Strategies to generate TESI began with a study by Vacanti et al., in which murine enterocyte preparations were seeded onto synthetic polymers [114]. Small intestine-derived enterocyte-seeded scaffolds were cultured for 4 days and remained viable and continued to proliferate. Direct omental implantation of these constructs in conjunction with partial hepatectomy induced engraftment and growth in 3 out of 23 implants with mature epithelial cell types and submucosal mesenchyme lining a neovascularized cystic structure with mucous and cellular debris within the lumen [114]. This pioneering study demonstrated the efficacy of combining primary cells with a synthetic scaffold as a viable approach to regenerating tissue with the potential for in vivo growth and development (Fig. 38.3). Vacanti's group later modified this approach to include mesenchymal cell types as well intestinal epithelium, and modifications of this approach now have much improved outcomes. We and others have demonstrated that the implantation of human and murine-derived OU embedded onto biodegradable scaffolds in immunocompromised mice results in successful growth of TES, TELi, TEE, TESI, TEC, etc. after 2–6 weeks [90,93–97,100,104].

By gross histology, TESI develops with a luminal facing epithelium and surrounding mesenchyme. The vascular supply is dually contributed to by de novo vascularization from donor endothelial progenitor cells and neovascularization from the host, and although capillary formation remains attenuated and rudimentary, the lymphatic network continues to grow [115,116]. The epithelial surface can range in architecture from a flat epithelium to alternating villus and crypt-like structures similar to mature native small intestine. Immunofluorescent staining for markers of mature epithelial and mesenchymal cell types demonstrates the ability of OU to restore ISCs, secretory and absorptive epithelial cells, and numerous mesenchymal support cells [95]. CBC cells retain their ability to proliferate as demonstrated by cellular division marker Ki67. At 4 weeks, human and murine TESI demonstrate the formation of microvilli with restoration of tight junctions, transporters, and functional brush-border enzymes in the correct apical and basolateral expression [93]. In addition, measurements of transepithelial resistance of rat-derived TESI show that while TESI has decreased active ion transport compared to native rat ileum, it does have similar epithelial barrier integrity [113]. Restoration of immunocyte populations is achieved only after TESI undergoes anastomoses to host bowel [117]. Although these data suggest that immune system maturation is dependent on luminal stimuli and time, they also show that engineered intestine can develop and recruit this crucial subpopulation of cells.

Numerous studies have investigated alterations to the protocol in order to enhance growth and function of TESI. Performing concurrent small intestinal resection stimulates intestinal adaptive changes as reflected by increased TESI size and diameter [107]. Anastomosis of TESI to native small intestine after the initial maturation period results in greater villus number, villus height, crypt number, and mucosal surface length, suggesting that mechanoluminal signaling factors may drive additional intestinal development [106,118]. The stimulatory signals have not been clearly identified but may include factors that are found at higher concentration in native intestine, such as VEGF, EGF, GLP-1/2, and other growth factors. FGF10 overexpression in a murine model generated heavier and larger sized TESI with more proliferating epithelia cells and longer villi and deeper crypts [119]. In addition, in a VEGF overexpression mouse model, submucosal capillary density was also improved, implying that neovascularization can be enhanced with the addition of growth factors [120]. Intestinal anastomosis and treatment with GLP-2 increases villus height and crypt depth in TESI, supporting the potential use of human-derived TESI as a useful model for testing emerging therapeutic

interventions aimed at stimulating structural and functional adaptation that may not be possible in controlled human clinical trials [121].

A significant limiting factor in the clinical translation of TESI is the need for generating a sizeable construct with a patent lumen that can be successfully anastomosed to the native intestine and can provide enough digestive and absorptive surface to allow the patient to gain weight and wean from PN. Animal models thus far have been encouraging. While rats with anastomosed TESI lost more weight initially than control rats without TESI, they regained weight more quickly and returned to 90% of preoperative weight by day 40 [98]. TESI generated from both murine and human OU has been demonstrated to recapitulate not only the structure of mature intestine but its digestive function as well. TESI bears active brush-border disaccharidases and demonstrate nutritional absorption [122]. Furthermore, both human and murine OU can be maintained in long-term culture without growth factor supplementation and these OU retain the ability to generate TESI with all mature intestinal epithelial cell types as well as ENS and mesenchymal cell types [123].

Pluripotent stem cell approaches—human intestinal organoids

Spence et al. defined a protocol to generate all of the components of the intestine except for the ENS from either human embryonic or iPSCs that were exposed to a temporal cocktail of growth factors, including Activin-A, FGF10, Wnt3a, Rspo, and others, to form spherical organoid structures with a luminal epithelium and surrounding mesenchyme, termed HIO [110,124]. In vitro, HIOs contain immature villus-like structures that lack lamina propria in their central core and fail to develop crypts with an extensive mesenchymal layer, as in mature intestine [110]. However, implantation into the kidney capsule and growth up to 16 weeks led to significant maturation of transplanted HIOs (tHIO) [108], demonstrating that the grafts have the capacity to differentiate in the presence of appropriate cues. Increased proliferation was observed in the intestinal crypts of tHIO, with restriction of Lgr5-positive ISCs and Paneth cells to the base of the crypts and mature absorptive/secretory epithelium throughout the villus, similar to native human small intestine. Several brush-border enzymes, such as sucroisomaltase, lactase, trehalase, and maltase-glucoamylase, had higher mRNA expression compared with in vitro HIOs and appropriate polarity was demonstrated by apical staining of sucroisomaltase, which was absent in in vitro HIOs. Moreover, intraluminal injections of small peptides were absorbed in tHIO. Increased organization of the mesenchyme occurred in tHIO, as platelet-derived growth factor receptor alpha-positive cells lined the basal side of villus epithelium and smooth muscle actin-positive myocytes condensed into a prominent band within the submucosa nearly identical to native human intestine. Compared to OU, the vasculature- and lymphatics-supporting tHIO are solely of mouse origin.

To test if tHIO respond to humoral factors and undergo intestinal adaptation as seen in postintestinal resection models of native intestine, mice transplanted with HIOs after 6 weeks then had an ileocecal resection with anastomosis and were harvested 1 week later. Similar to OU, significant intestinal adaptation occurred in tHIO following postileocecal resection as demonstrated by increased villus height/crypt depth, crypt fission, and thickness of the smooth muscle compared to surgical sham tHIO. These data suggest that HIOs respond to intrinsic cues that augment intestinal adaptation. Although tHIOs display significant features of maturation beyond that observed in HIO in culture, comparative unsupervised hierarchical clustering of RNA-sequencing data of tHIO compared to human fetal and adult small intestine demonstrated that tHIO more closely resemble fetal small intestine rather than mature adult small intestine [125]. The implantation of HIO-seeded PGA/PLLA scaffolds into the omentum of immunosuppressed mice, similar to OU, has yielded promising results: generating HIO-TESI after 3 months that is nearly indistinguishable to native intestine [104].

Although HIOs provide a potential platform for generating limited amounts of patient-specific intestinal tissue, they lack a critical element intrinsic to OU that are required for peristalsis, an ENS. There are two approaches to derive enteric neural crest cells and their derivatives: from primary intestinal biopsies and directed differentiation of hPSCs (Fig. 38.3) [126–129]. Coimplantation of HIO-TESI with murine OU repopulates cell types of the ENS as demonstrated by nerves and glia adjacent to HIO-derived epithelium and within the myenteric layers [104,130]. HIOs cocultured with vagal neural crest cells derived from hPSCs for 4 weeks and implanted into the kidney capsule demonstrate immature restoration of ENS components [131]. Submucosal and myenteric ganglia are restored with retention of inhibitory neurons; however, excitatory choline acetyltransferase was lost after transplantation and restoration of a neuroepithelial circuit necessary for transmission of intraluminal sensory information was not restored as neurons lacked synaptic formation with neuropods of enteroendocrine cells. Applying an alternate differentiation protocol to generate the PSC-derived neural crest cells, we were able to retain important ENS subtypes and functional contraction of HIO-TESI with restoration of key components of the ENS, including neuroepithelial connectivity and neuron-dependent smooth muscle contractility and relaxation [130].

Remaining barriers to the generation of tissue-engineered intestine

Several barriers remain to be overcome before intestine can be routinely tissue-engineered from stem and progenitor cells. First, tissues grown from stem/progenitor cells often form disorganized tissue of a more primitive histologic type. TESI from multiple donor origins contains a spectrum of tissue organization, with only a portion recapitulating organized crypts and villi. Second, although generation of an entire organ may not be necessary in order to replace sufficient function, tissue of adequate size and development must be generated. This will require upscaling protocols, large animal models, and perhaps the addition of small molecules that will be acceptable to regulatory bodies. Third, as the volume of tissues generated increases, the issue of blood flow must be addressed, either through concomitant angiogenesis or via an engineered or autologous graft. Furthermore, the first in human clinical trials must proceed with thoughtful and thorough regulatory and ethical guidance, and data should be derived from all attempts to translate by careful reporting. There is a tremendous opportunity for TEI therapies to be appropriately studied and reported through preregistered trials.

Conclusion

Multiple populations of stem/progenitor cells have been identified in the intestinal crypts, including rapidly cycling Lgr5+ CBC cells, a quiescent +4 population, and/or progenitor and differentiated cells capable of reverting to a stem phenotype. Any of these may have the potential to generate de novo intestine. The stem cell niche is maintained by a complex network of signaling molecules which, although not completely understood, involves a modulated interaction of multiple factors, including Wnt, ErbBs, Notch, Hedgehog, and BMP. Advances in understanding ISC and the ISC niche have offered possibilities to harness these regenerative processes for the generation of TEI. TESI has been generated from multiple sources of stem/progenitor cells such as OU from intestinal biopsies or HIO from iPSC. While multiple barriers still exist, there has been rapid progress toward future clinical applications of TEI based on foundational principles discovered in developmental biology and rigorous scientific study of an elegant stem cell niche.

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Chapter 39

Liver stem cells

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Introduction

The liver is a complex organ that performs a wide range of different metabolic processes essential for body function [1]. Although liver possesses unique regenerative capacities, upon serious injury scarring occurs, and liver repair is often compromised. Significant loss of liver function may lead to serious consequences for the patient [2–4]. Currently, orthotopic liver transplantation is the only effective treatment for acute or end-stage liver failure. However, the decreasing number of organ donors and complications associated with lifelong immunosuppression requires us to develop alternative therapies for patients [5].

Donor adult hepatocytes have been successfully transplanted to treat metabolic liver diseases [6]; however, the graft is eventually cleared by the immune system and is further compounded by the scarcity of the liver tissue [7]. Therefore new sources of liver tissue such as adult progenitors and pluripotent stem cell (PSC)–derived somatic cells represent attractive cell-based systems to support failing liver function in humans and to better model liver biology "in a dish."

Liver architecture and function

The liver is the largest internal organ providing different metabolic, endocrine, and exocrine functions such as drug detoxification, glycogen storage, production of serum proteins, and bile secretion [1,8]. The organ is composed of four lobes that are further divided into lobule structures—the basic architectural unit of the liver. Liver lobules are hexagonal in appearance, with each corner demarcated by the "portal triad," consisting of the portal vein, bile duct, and hepatic artery [8]. Hepatocytes are the parenchymal cells of the liver that make up approximately 70%–80% of the organ mass and carry out most of the metabolic functions. Hepatic function is supported by the nonparenchymal cells (NPCs) that consist of numerous cell types such as cholangiocytes, sinusoidal endothelial cells, natural killer cells, Kupffer cells, and stellate cells [9]. Hepatocytes are highly polarized epithelial cells, where their basolateral surface is directly connected with sinusoidal endothelial cells to facilitate the exchange of materials between hepatocytes and blood vessels. Tight junctions between hepatocytes allow for the canaliculus formation. The bile canaliculi collect bile salts and acids that are transported across the apical side of hepatocytes to bile ducts, where they are finally stored in gall bladder before release into duodenum [10].

Liver development

Several animal studies have proven regulatory pathways during liver organogenesis to be evolutionary conserved [11]. During gastrulation the endoderm germ layer is established and forms a primitive gut tube that is further subdivided into foregut, midgut, and hindgut regions [11]. The foregut gives rise to hepatic diverticulum, where the anterior portion of it forms liver and posterior portion allows formation of the gall bladder and extrahepatic bile ducts. Liver specification is induced by fibroblast growth factor (FGF) signaling from the cardiac mesoderm and bone morphogenetic protein (BMP) signaling from the septum transversum mesenchyme (STM) [12–14]. Once hepatic specification is established, hepatic epithelium pseudo stratifies, delaminates and the newly formed hepatoblasts invade the STM, where they recruit mesenchymal cells prior to liver bud formation [15]. Once specified, the bipotential hepatoblasts express markers of fetal (AFP) or adult hepatocytes (HNF4α, HNF6, and ALB) and biliary epithelium (CK7 and CK19) [8]. Hepatoblast differentiation toward hepatocytes versus biliary epithelial cells (BECs) is regulated by an activin/TGF beta-signaling

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gradient [16–18]. Furthermore, upon colonization of the nascent liver bud, hematopoietic cells secrete oncostatin M, which in combination with glucocorticoid hormones, hepatic growth factor (HGF), and Wnt promotes hepatocyte differentiation, proliferation, maturation, and liver zonation [19–22]. Regulatory signals from periportal mesenchyme such as Wnt, TGFB, and Notch are involved in biliary development [8]. In response to mesenchyme signals, bipotential hepatoblasts downregulate hepatic factors (HNF4α, Tbx3, C/EBP) and increase expression of transcription factors, such as HNF6 and HNF1β, to stimulate cholangiocyte formation [16,23–25].

Fetal liver stem cells

Several studies using different culture systems have demonstrated the evidence for the presence of a potential liver "stem cell" in the fetal liver, which has a capacity for unlimited proliferation and multilineage differentiation [10]. Due to their unique features, it has been suggested that hepatic stem cells (HpSCs) may become an alternative cell source to primary hepatocytes for future clinical applications [26]. Therefore several studies have tried to isolate, purify, and characterize liver stem cells from human and rodent livers using flow cytometry. It has been demonstrated that isolated HpSCs expressed markers of stem cell origin such as CD13 [27], CD29 and CD49f [28], CD133, CD117, Epcam [29], and DLK [30], markers of hepatocyte and cholangiocytes [31] as well as markers of both hematopoietic and mesenchymal stem cells (MSCs) [32,33]. Recently, Zhang et al. [26] have identified CUB domain-containing protein 1 (CDCP1) to be an important marker for HpSCs isolation from the human primary fetal liver. Flow cytometry permitted the isolation of the $CDCP1^{+}CD90^{+}CD66^{-}$ subpopulation that exhibited remarkable clonal expansion and self-renewal capability. Moreover, in vivo studies demonstrated that this subpopulation repopulated over 90% of the mouse liver and differentiated to metabolically functional hepatocytes 1 month after transplantation. Dan et al. [34] reported the isolation of a population of human fetal liver multipotent progenitor cells called "hFLMPCs" that exhibited capacity to differentiate to liver cells and repopulate in a mouse liver injury model. These cells expressed several stem cell-related markers such as CD90, c-Kit, CD44h, and EpCAM but did not express hepatic markers such as AFP or ALB. This was further confirmed by Schmelzer et al. [32]. Recently, the same group [35] isolated a similar subpopulation of cells expressing mixed endodermal–mesenchymal markers, as had been reported by other groups [36,37]. The cells were capable of differentiating into mesenchymal lineages of bone, cartilage, endothelium, and fat. Furthermore, upon engraftment into a mouse $Rag2-/-\gamma c-/-$ model, cells acquired de novo progenitor state and were able to proliferate and differentiate into functional hepatocytes in vivo.

Although human HpSCs from fetal and postnatal human liver have been demonstrated to self-replicate and differentiate to adult hepatic tissue [26,32], the source of these primed stem cells is still limited and is not available commercially. Furthermore, use of these cells for hepatocyte transplantation is still questionable due to their origin; and therefore long-term safety assessments should be performed in order to determine their potential off-target effects.

Hepatocytes and liver progenitors in organ regeneration

The liver is a remarkable regenerative organ; however, the cellular source for hepatocyte regeneration in injured parenchyma remains complex. It has been demonstrated that during liver homeostasis or its mild injury, regeneration occurs through self-replication of hepatocytes [38–40]. However, under severe liver injury conditions, when parenchymal cell proliferation is impaired and high levels of senescence are detected, liver progenitor cells (LPCs) derived from BECs play an essential role in liver regeneration [41].

Molecular signaling and processes involved in liver regeneration

Liver regeneration is a complex process, with different molecular signaling pathways playing key roles in orchestrating organ regeneration. It has been widely documented that involvement of Wnt/β-catenin signaling is essential for the liver regeneration in zebrafish, rodents, and humans (see Ref. [40] review for details). During the early stages of partial hepatectomy (PHx), β-catenin translocates to the nucleus, where it activates essential proliferative genes such as cyclin D1. This has been confirmed by ablation or knockout of β-catenin, which severely delays liver regeneration [42,43]. Furthermore, R-spondin-1 (Rspo-1), strongly potentiates canonical Wnt-signaling pathway by acting as a ligand for leucine-rich repeat-containing G protein coupled receptors 4–6 (LGR4–6), promoting liver regeneration [44]. One of the most important signaling pathways is the Hippo/Yes-associated protein 1 (Yap) that orchestrates liver homeostasis by regulating organ size and cell fate [45,46]. Furthermore, Yap has been demonstrated to be a downstream effector of the Hedgehog pathway, resulting in increased proliferation of hepatocytes and stellate cells under liver injury conditions [47]. Recent studies by Li et al. [48] reported that chromatin remodeling gene

Arid1a endows hepatocytes with a competence to respond to the Hippo/Yap signal, which activates the expression of liver-progenitor-like cell genes and renders them competent to respond to injury-associated regenerative signals. In support of this, Planas-Paz et al.'s [49] CRISPR screen identified that contrary to RSPO-LGR4/5-mediated WNT/β-catenin activity, mTORC1 and YAP signaling are important in promoting ductular reaction and liver regeneration. Hedgehog signaling has been reported to induce Gli transcription factors that activate target genes responsible for regulating cell proliferation, survival, and differentiation [50,51].

Recently, it has been described that among factors released immediately after PHx, different prosurvival antiapoptotic functions are activated during liver regeneration process [52]. Several studies reported that cellular oxygen sensor [52], bile acids (BA) [53,54], and CCAAT/enhancer binding protein [55] are important factors that promote liver repair and impairment of these factors may have deleterious effects on liver regeneration. Furthermore, Liu et al. [56] and Wahlström et al. [57] demonstrated the impact of microbiota on liver regeneration. Although the interplay between microbiota and BA is not well understood in the process of organ regeneration, it has been hypothesized that gut microbiota regulates BA homeostasis. Therefore further research on probiotics may open a new therapeutic opportunity and allow for further understanding of the processes involved in liver disease and regeneration [40].

Hepatocytes' role in liver regeneration

Although hepatoblasts are precursors for functional hepatocytes and cholangiocytes during liver development, their existence post birth is questionable. Therefore adult liver maintains homeostasis by division of hepatocytes [38,39]. It has been reported that hepatocytes are functionally heterogeneous, and metabolic zonation determines different hepatic populations capable of proliferating and self-renewing to maintain homeostasis (see review by Ref. [40]). Lineage-tracing experiments in mice identified a population of proliferating and self-renewing hepatocytes in the centrilobular zone 3. These cells, expressing early liver progenitor markers such as Axin2 and Tbx3, were able to recover one-third of the mouse liver lobule [38]. Although promising, recent studies reported the lack of evidence of a hepatocyte niche around the central vein that would be responsible for liver homeostasis; therefore a more detailed analysis is required to probe these differences. Furthermore, Font-Burgada et al. [58] identified a population of Sox9/HNF4α-positive hepatocytes in the periportal zone. These hybrid hepatocytes were able to replenish liver mass upon damage; however, they were not participating in normal liver homeostasis. Recent lineage-tracing studies discovered periportal Mfsd2a [59] and telomerase [60] expressing hepatocytes were capable of recovering liver mass after chronic injury or able to generate small clones scattered throughout the liver lobule following transplant.

Cholangiocytes and liver stem cells in liver regeneration

Although hepatocytes are capable of proliferation during homeostasis or even after surgical removal of two-thirds of the liver (PHx) [10,61], the regenerative capacity of these cells is significantly reduced in the presence of noxious agents. Despite toxic injury, the liver is still capable of regeneration and led to the theory of an adult stem/progenitor compartment ([62,63]; see review by Ref. [64]). It has been widely believed that these facultative progenitors are derived from a subpopulation of the duct/biliary cells (oval cells) from the liver compartment [65]. The early studies have reported that cholangiocyte-like oval cells, upon liver injury, become liver stem cells capable of regenerating both hepatocytes and cholangiocytes [66]. Several groups have indeed reported similar results, where oval cells expressing Epcam, FoxL1, Lgr5, or Sox9 have been regarded as bipotential facultative stem/progenitor cells [67–71]. This theory has been further supported by experimentation demonstrating that during hepatocyte death and senescence, bile duct–derived progenitor cells contribute to liver reconstitution in vivo [72]. More recently, Raven et al. [41] confirmed these findings and demonstrated that blocking hepatocyte proliferation, by overexpressing p21 or inhibiting Itgb1, resulted in the generation of new hepatocytes from biliary epithelia.

Although the theory of hepatocytes being derived from liver stem cells of bile duct origin has been widely accepted by the field, certain studies have questioned this rationale. Several recent lineage-tracing approaches failed to support stem cell progenitor existence and demonstrated that the repair of damaged liver relies predominantly on the hepatocyte compartment [39,73]. These differences in cell plasticity are important and are likely the product of the environment created in vivo [74]. Studies by Yimlamai et al. [46] have reported that downregulation of Hippo signaling efficiently converted hepatocytes to panCK + /Sox9 biliary cells and MIC1-1C3 + oval cells. Recently, Schaub et al. [75] demonstrated a successful transdifferentiation of hepatocytes to cholangiocytes by TGF-β signaling without the need for Notch activity. These results provided evidence of hepatocyte plasticity with a formation of stable biliary system that might reveal opportunities for the treatment of Alagille syndrome (ALGS) and other cholestatic liver diseases. What is more, Deng et al. [76] further confirmed direct

lineage conversion without the progenitor status. The studies described that BECs largely contribute to hepatocyte regeneration upon chronic liver damage. The converted BECs gained an HNF4α + CK19 + biphenotypic state and replenished ~55.7% of the liver parenchyma upon 3.5 diethoxycarbonyl-1,4-dixydrocollidine (DDC) treatment. The authors discussed that former studies on bile duct–derived progenitor cells used relatively short-term liver injuries, which could not assess whether NPCs or BECs contribute to hepatocyte regeneration. Furthermore, the study by Deng et al. [76] indicated that Sox9 expression does not distinguish BECs from LPCs, and markers of progenitors were not detected during cholangiocyte-to-hepatocyte conversion as previously published in Raven et al. [41] studies.

Pluripotent stem cell–derived hepatoblasts and hepatocytes

Although organ transplantation is the only longterm treatment for end-stage liver disease and inborn errors in metabolism, availability of donors is severely limited; hence, only a minority of eligible patients will receive a transplant [77]. Therefore liver cell transplantation is an alternative option with a potential to cure congenital liver diseases as well as acute-on-chronic liver failure. As such treatment is considered minimally invasive, it has several advantages over the whole organ transplantation such as faster recovery time and cost savings [77]. Although preliminary clinical studies on primary hepatocytes as well as hematopoietic and MSCs provided evidence of their potential suitability for cell engraftment [78,79], longterm expansion capacity of these cells led to search for a new source of engraftable and functional liver cells.

In addition to transplantation, a renewable source of liver tissue for modeling human response "in a dish" is vital to model disease, repurpose drugs and improve the efficiency of the drug development process. Recent research analyses indicated that drug development takes over 12 years and requires a cost of $2.6 billion [80,81]. Unfortunately, high percentage of drug failure during clinical phases II and III leads to significant drug attrition during the development process. As toxic effects in the liver are usually detected at later stages of the drug development, there is a need to improve screening platforms and preclinical models [82,83]. Currently, industry heavily relies on different types of cell-based systems. Primary human hepatocytes (PHH) are considered as a gold standard model to study drug metabolism; however, their rapid loss of phenotype, isolation from transplant rejected organs, and loss of function limit their use for long-term drug toxicity studies in vitro [84]. Therefore researchers have searched for more accessible and cheaper alternatives such as cancer-derived cell lines. The immortalized lines such as HepG2, Huh-7, Hep3B, Fa2N-4, and HepaRG have been widely used in the industrial setting displaying a moderate potential to metabolize different drugs [85]. Despite having advantages, their cancer origin offers limited biological relevance when compared to primary organ or cell type. Therefore stem cell technology has been proposed as a suitable alternative to overcome limitations associated with primary hepatocytes or cancer cell lines (see review by Ref. [86]). Human PSCs (hPSCs) include human embryonic stem cells (ESCs) (hESCs) and human-induced PSCs (iPSCs) (hiPSCs), have the ability to self-replicate and differentiate into all types of body cells, including functional hepatoblasts and hepatocytes [87–92]. Therefore hPSC-derived hepatoblasts or hepatocyte-like cells are an attractive cell source to provide unlimited number of functional hepatocytes for liver transplantation and improved in vitro modeling.

Research has produced a number of efficient hepatocyte differentiation procedures from PSCs using different growth factors or small molecules [5,93–107]. Recent advances in generating functional hepatocytes from stem cells allows one to model different diseases such as metabolic disorders [108,109], nonalcoholic liver diseases [110,111], and drug or tobacco-induced liver injuries [112,113]. Although stem cell–derived hepatocytes have been useful for drug toxicity studies and disease modeling, further improvements of these models are required to understand cell–cell interactions or cell–ECM signaling that usually affects liver functions in vivo [86]. Several engineering tools such as biomaterial scaffolds [114,115], cellular microarrays [116], protein micropatterning [117–119], microfluidics [120], and bioprinting (see review Ref. [121]) have been developed to control cellular microenvironment and hepatic niche (Fig. 39.1).

Despite advances in hepatic differentiation protocols, the procedures are often considered as time-consuming, expensive, and generate mixed populations of hepatocytes and other cell types [134]. Indeed, recent genome-wide expression studies demonstrated significant differences in gene expression between stem cell–derived hepatocytes and their target cells (i.e., primary hepatocytes) [101,135]. Distinct gene expression and epigenetic signatures were also observed between hepatocytes generated from the same donor using different strategies [136]. Therefore application of Omics technologies is essential to generate high fidelity hepatocytes for cell based modelling [137,138].

Studies have focused on the generation of LPCs from PSCs. Although potentially promising, efficient methodologies for maintaining hepatoblasts or LPCs under suitable conditions in vitro remain challenging. Several groups have reported an establishment of proliferative PSC-derived LPCs that were capable of differentiation to

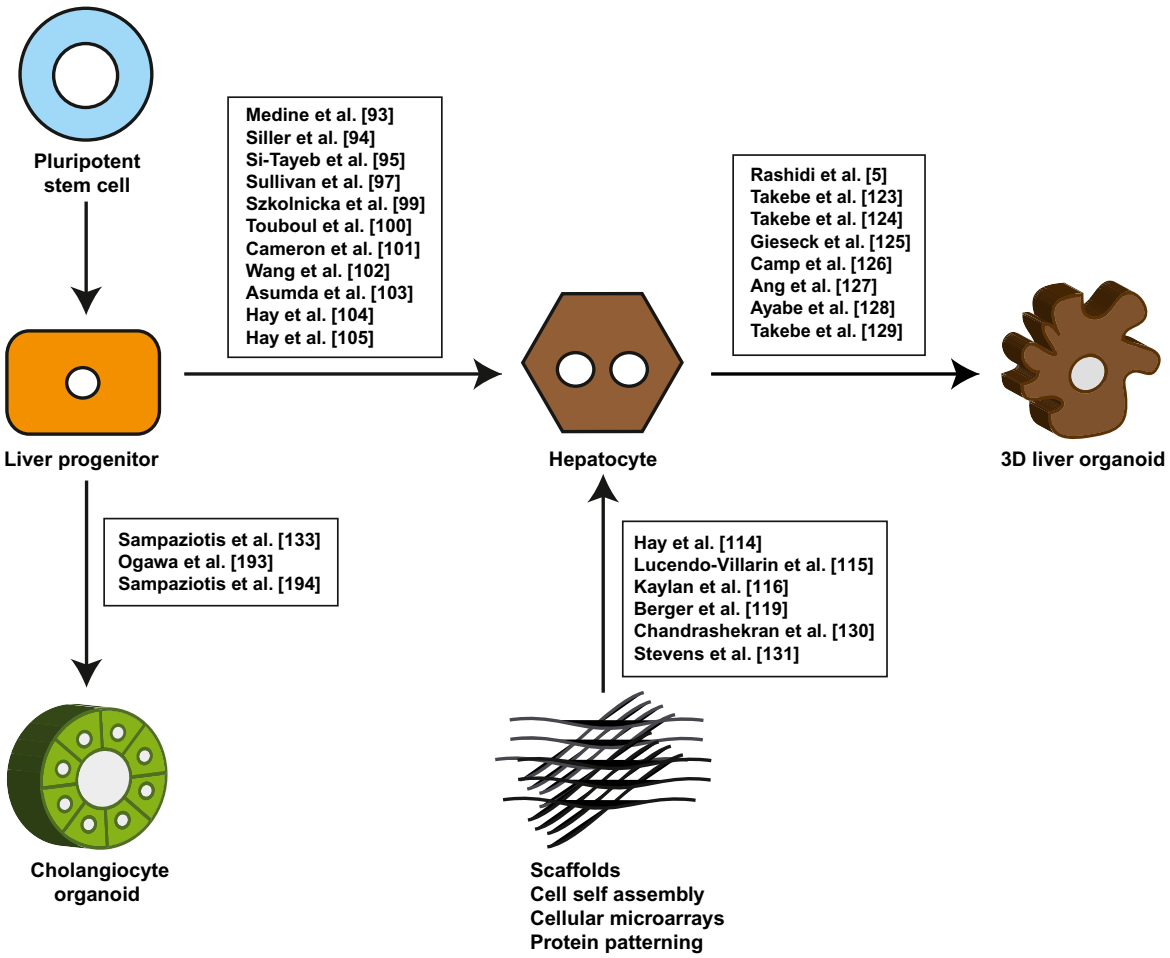

FIGURE 39.1 Established hepatic and cholangiocyte differentiation protocols from pluripotent stem cells: liver progenitor→hepatocyte—for details see Refs. [93–95,97,99–103,105–106,122]; hepatocyte→3D liver organoid—for details see Refs. [5,123–129]; scaffolds cellular microarrays protein micropatterning→hepatocyte—for details see Refs. [114–116,119,130,131]; liver progenitor→cholangiocyte organoid—for details see Refs. [133,193,194].

both hepatocytes and cholangiocytes [139,140]. However, to isolate and characterize these cells, a combination of cell surface markers was used, which were not specific to the liver [141]. To improve LPCs isolation and expansion, several studies have used specific extracellular matrices or cell surface markers. Takayama et al. [142] generated PCS-derived LPCs on human recombinant laminin 111 (LN111) matrix. Cells cultured on LN111-coated dish were maintained for 3 months in vitro as a bipotential population. Although promising, the authors applied adenovirus-mediated gene transfer to generate hepatic lineage that may also induce changes in the host cell. Two years later, Kido et al. [143] identified carboxypeptidase M as a novel marker to isolate hiPSC-derived LPCs that demonstrated self-renewal capacity and bidirectional differentiation. Furthermore, Zhang et al. [144] generated self-renewing hepatoblasts from hESCs by a stepwise induction strategy. The human hepatoblasts were successfully maintained in a specific ECELS medium supplemented with epidermal growth factor, glycogen synthase kinase-3 inhibitor (CHIR99021), transforming growth factor B receptor inhibitor (E-616452 or SB431542), lysophosphatidic acid, and sphingosine 1-phopshate [145]. Upon specific hepatic signals, hepatoblasts were able to differentiate to mature hepatocyte and displayed bile duct-like structures in Matrigel-embedded 3D culture.

3D liver organoids and expansion

Despite extensive investigation and optimization of different cell culture systems, the long-term in vitro amplification of hepatocytes remains very challenging due to the loss of their features and their proliferation capacity in culture. Tissue engineering has received a lot of attention and opened new perspectives to develop new culture cell models to improve their stability and functionality in vitro. Hepatocytes derived from PSCs, either from

iPSCs [95,146,147] or hESCs [101,105,106,122,148], have shown great promise. Although the results obtained with stem cell–derived hepatocytes have been encouraging, the cost and time needed to maintain and differentiate cells into a particular lineage are some of the limitations that restrict the current use of these cells for clinical applications. What is more, the widely used 2D hepatic differentiation protocols do not recapitulate organ morphogenesis or tissue organization, which can compromise their application [5]. Therefore novel strategies such as 3D culture systems have been generated to reconstitute more faithfully the in vivo hepatic environment that would improve long-term maintenance and large-scale production of cells.

Pluripotent stem cell–derived liver organoids

During liver development, progenitor liver cells delaminate and migrate into the STM to form highly self-organized and vascularized liver bud [15,149]. As the process of formation and growth of the hepatic diverticulum requires various signals from endothelial and mesenchymal cells, understanding the key-cross signaling pathways among cells is essential to fully comprehend hepatogenesis and function [150]. To generate 3D functional liver tissue iPSCs-derived hepatocytes, human umbilical vein endothelial cells, and MSCs were aggregated [123,124]. Although the authors were able to generate liver tissue that mimics the in vivo equivalent, the system was not suitable for scaled-up production. Alternative approaches to generate functional liver tissue and to better understand multilineage communication have also been developed by Gieseck et al. [125], Camp et al. [126], Ang et al. [127], Ayabe et al. [128], and Lucendo-Villarin et al. [92]. However, in order to produce stable liver spheres or organoids for application, challenges associated with scale and reproducibility have to be overcome. Recently, to facilitate the future therapeutic application of organoid-based approaches, Takebe et al. [129] established reproducible, functional and vascularized human liver buds from human iPSCs. Such studies provide an advance in the quest to build human liver tissue for the clinic and to study disease. More recently, Rashidi et al. [5] developed a defined 3D hepatosphere system that permitted the specification and culture of functional liver tissue for over a year in vitro, provided liver support in vivo, even in immunocompetent recipients. Although challenges still exist, these studies have opened up new experimental avenues for human translational medicine.

Bile duct–derived organoids

While PSC-derived hepatocytes hold a great promise for translational applications, further safety and optimization of 3D differentiation protocols are required before they are ready for the clinic [151,152]. Therefore parallel studies have focused on the stable expansion of nontransformed cell types for regenerative therapies and in vitro modeling (Fig. 39.2). Several studies have demonstrated that liver displays a remarkable regenerative capacity, where different modes of response occur depending on the type of damage. Lineage-tracing studies have demonstrated that under steady-state conditions in vivo, Axin + pericentral hepatocytes are able to self-renew and proliferate [38,60]. What is more, upon PHx, hepatocytes are capable of regenerating the whole liver within 2 weeks after damage without dedifferentiation into progenitor/stem cell–like state [10]. However, under severe or chronic, the stem cell compartment is activated [66]. Several studies have described the generation of long-lived 3D liver spheres that mirror oval cell in terms of their plasticity [71,153]. Under specific Rspo-1 growth-factor conditions, single mouse Lgr5 + liver stem cells were demonstrated to be clonally expanded as organoids for over multiple months and could be induced to functional hepatocytes both in vitro and in vivo [71]. Soon after, Epcam + bile duct–derived bipotent progenitor cells from human liver biopsies were established and displayed long-term proliferation and genetic stability. Furthermore, using this technique, the authors generated 3D organoids from patients with alpha 1-antitrypsin deficiency and ALGS and were able to model the effects of those mutations in vitro [153].

Hepatocyte-derived organoids

The work of Huch et al. [71,153] has greatly improved liver organoid formation from BECs and primary liver cancer (PLC) cells [157]. Despite progress, it has been challenging to culture and stably expand healthy mature hepatocytes in vitro from cholangiocyte-derived organoids. To overcome this issue, several studies have described generation of long-term culture system for proliferative hepatocyte spheroids. It has been reported that hepatocyte-derived progenitors generate 60 times more hepatocytes than progenitors derived from cholangiocytes [159]; therefore use of solely primary hepatic tissue could be a preferred source to establish 3D models for in vitro expansion. Several studies have described the importance of injury-invoked regenerative signals during liver damage [160,161]. Recently, Peng et al. [151] generated long-term 3D mouse organoids by inducing tumor necrosis factor α (TNFα). The injury-induced inflammatory cytokine secreted by Kupffer cells has been reported to play an essential role in initiating liver regeneration [160]. Therefore given its role in tissue repair, the authors asked whether such inflammatory signals could promote primary hepatocyte proliferation. Single-cell RNA sequencing (RNA–seq) studies on 3D mouse organoids revealed a

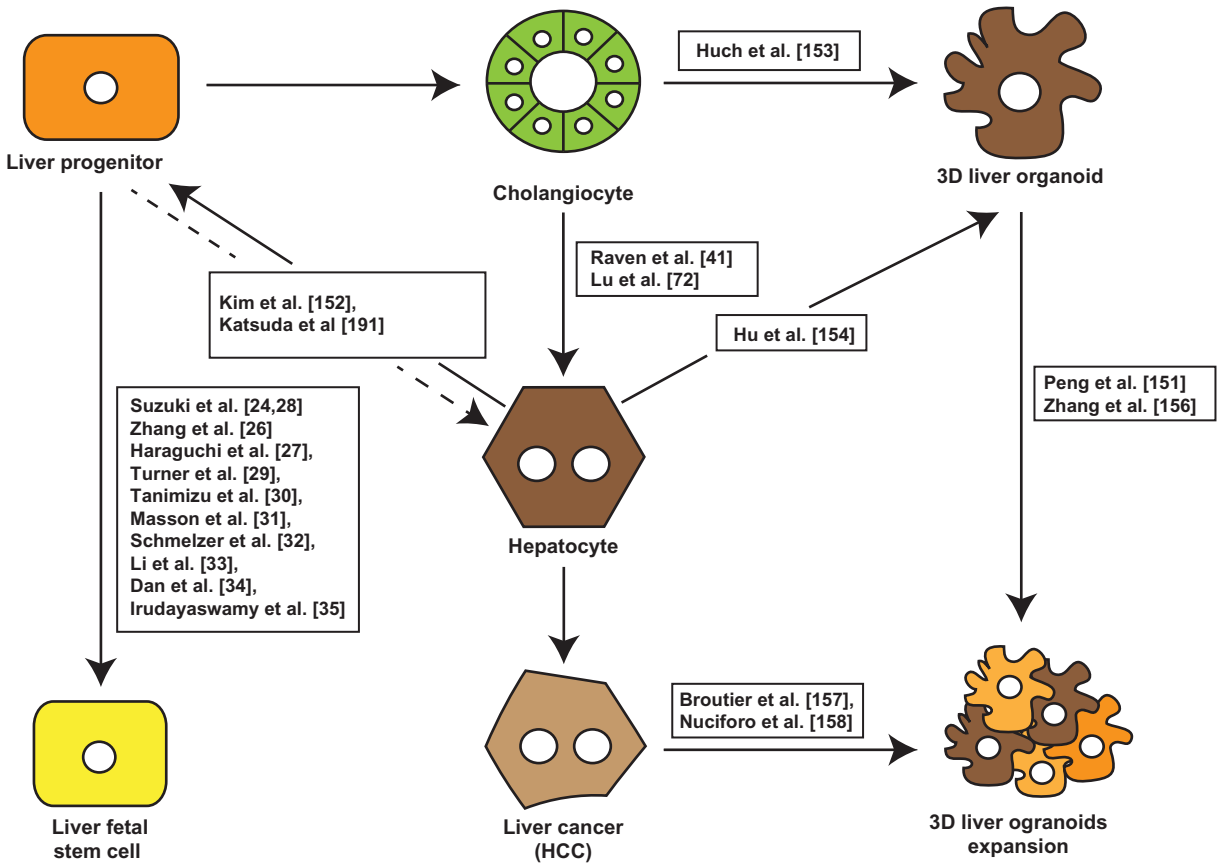

FIGURE 39.2 Recent advancements in generating hepatic cells from primary liver tissue: cholangiocyte→3D liver organoid—for details see Ref. [153]; cholangiocyte→hepatocyte—for details see Refs. [41,72]; hepatocyte→3D liver organoid—for details see Ref. [154]; hepatocyte→liver progenitor—for details see Refs. [152,191]; liver progenitor→liver fetal stem cell—for details see Refs. [24,26–28,30–35]; 3D liver organoid→3D liver organoids expansion—for details see Refs. [151,156]; liver cancer (HCC)→3D liver organoids expansion—for details see Refs. [157,158].

broad expression of hepatic but not biliary markers, and cells extensively repopulated injured liver of FAH −/− mice. Therefore harnessing tissue repair signals may be a powerful approach to manipulate hard-to-culture cell types for different medical implications. The same year, Hu et al. [154] described successful generation of long-term 3D organoids derived from mouse and human primary hepatocytes. The authors reported establishment of hepatic 3D system from single hepatocytes that were capable of growing for multiple months in vitro, retained key morphological and functional features as well as repopulated damaged liver in Fah −/− NOD Rag1 −/− Il2rg −/− (FNRG) mice. It has been suggested that the combination of the two protocols [151,154] could be essential for further clinical development. Lastly, remarkable in vitro expansion of PHH was achieved by Zhang et al. [156] using defined medium conditions. The authors generated proliferating human hepatocytes (ProliHHs) that displayed both hepatic and progenitor features could be serially passaged and expanded by 10,000 fold. Bipotent ProliHH organoids could be reverted to mature phenotypes and were able to repopulate damaged mouse liver up to 60%. The work of Zhang et al. provides an efficient protocol for large-scale production of cells suitable for treating human liver disease and drug metabolism studies.

Novel scaffolds for liver organoids

Although promising, those techniques often suffer from limitations that do not allow the use of these cells for clinical application. The use of animal-derived components such as Matrigel cannot be used for large-scale production under good manufacturing practice conditions. To overcome the problem of xeno-derived components, novel engineered scaffolds [162], naturally occurring [163] or synthetic material [164], are under scrutiny to improve organoid-differentiation. In the future the use of biocompatible scaffolds in combination with organoid-based systems could provide a safe and efficient source of functional cells for transplantation until the patient

receives an organ transplant or the native liver recovers. Recently, Chandrashekran et al. [130] generated liver organoids from human umbilical cord–derived MSCs and human cadaveric donor–derived hepatocytes that were cultured on methylcellulose scaffolds. Methylcellulose is a synthetic chemical product derived from cellulose [165] and is widely used in industry and clinical settings [166–168]. Although the authors demonstrated the methylcellulose-based liver organoids to be functional in vitro, the results obtained were still significantly lower than of Matrigel cultures. Nevertheless, establishment of liver organoids in methylcellulose and further encapsulation of these aggregates in alginate-methylcellulose 3D scaffolds may enable clinical grade expansion and may be highly applicable in the treatment of acute liver failure in the future. In order to tackle tissue architecture and scale issues, Stevens et al. [131] took a different approach and developed SEEDs (in situ expansion and engineered devices). This approach used primary parenchymal, vascular, and stromal cells and arranged those in a specific architecture in the biodegradable hydrogel. The bioencapsulated organoids expanded in vivo in response to regenerative cues, expanding up to 50-fold in animals with liver injury. Recent studies by Rashidi et al. [5] have provided evidence for successful subcutaneous implantation of scaffold-based 3D liver organoids. The FDA-approved material, polycaprolactone, was selected to develop an implantable liver graft that demonstrated vascularization and function upon transplantation into immunocompetent and deficient recipients. The implantation of 3D hepatocytes–loaded scaffolds resulted in significant body weight recovery and increase in hepatic functions over 14 days in vivo. As subcutaneous transplantation of the scaffold is a simple procedure, it may reduce the need for general anesthesia and invasive abdominal operations in the future to treat failing liver function in humans.

Organoids as a model to study liver cancer disease

Currently, hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) represent the most common PLCs in the world [169]. Despite much progress in drug research [169,170], most of the cancer drugs fail to meet clinical end points in phase III trials [171]. Recently, the newly developed multikinase inhibitor regorafenib [172] and monoclonal antibody nivolumab [173] have been demonstrated to display efficacy for advanced HCC. However, the effective treatments against PLCs are still limited; therefore new anticancer therapies are urgently needed. The development of new human models that recapitulate key features of tumors such as their tissue structure and cellular heterogeneity are essential in these endeavours [158,174]. The development of new organoid-based technologies has aided cancer research, studying prostate [175], pancreatic [176], colorectal [177], breast [178], and bladder [179] cancers. Recently, Broutier et al. [157] described a new organoid culture system to propagate human PLC–derived organoids that would be suitable for disease modeling and drug screening. The 3D organoids were derived from patients suffering from HCC, CC, or combination of both tumors and preserved their histological architecture, gene expression and could be expanded over time in vitro that allowed to identify an ERK inhibitor SCH772984 as a potential therapeutic agent against PLCs. A year later, a similar study performed by Nuciforo et al. [158] generated long-term organoid cultures from tumor needle biopsies of HCC patients and demonstrated sorafenib inhibitor sensitivity. These studies evidence the importance of 3D systems in drug development and establishing personalized medicines.

Reprogramming of human hepatocytes to liver progenitors using different culture conditions

Currently, hepatocyte transplantation represents the only possible alternative to whole liver transplantation for patients with end-stage liver diseases. Although promising, cell transplantation results in a partial and relatively short-term correction of liver dysfunction. Furthermore, it has been severely hampered by different issues related to shortage of donor tissue, insufficient hepatocyte proliferation in vitro, or low engraftment efficiency of hepatocytes in vivo. To overcome these issues and to further develop cell-based therapies, much effort has been directed toward the potential use of stem cells capable of self-renewal, including ESCs, iPSCs, MSCs, and LPCs [101,147,180,181]. Despite the great promise of stem cell–derived hepatocytes in the field of regenerative medicine, their use in clinic is impeded by their differentiation potential [96], poor engraftment [182], likelihood of immune rejection [183], or the risk of cancer development [182,184,185].

Recently, several groups have reported that adult mature hepatocytes can be reprogrammed into bipotent and proliferative LPCs in response to chronic liver injury [39,46,159,186]. Therefore conversion of mature hepatocytes to LPCs could serve as a novel cell source that can be used in liver regenerative medicine [6] and would allow one to further understand molecular processes involved in cell reprogramming during liver disease/dysfunction. Recent developments in small molecules have successfully replaced the use of growth factors and contributed to the induction and maintenance of stem cells as

well as to their differentiation into distinct tissue lineages [94,187–189]. Kawamata and Ochiya [155] have identified that a combination of four small molecules Y-27632 (Rho-associated kinase inhibitor), PD0325901 (mitogen-activated protein kinase inhibitor), A83-01 (type 1 transforming growth factor−β receptor inhibitor), and CHIR99021 (glycogen synthase kinase-3 inhibitor) allowed for stable culturing of multiple types of rat stem/progenitor cells, including ESCs, CD63 + /Ck14 + mammary gland progenitor cells, and multipotent mammary tumor cells. The positive results from this study have led to the generation of chemically induced liver progenitors (CLiPs) from mature rat hepatocytes using the same four-molecule cocktail [190]. CLiPs displayed stable expression of liver stem cell features in long-term culture in vitro and were able to differentiate both to hepatocytes and functional cholangiocytes, as well as extensively repopulate chronically injured liver tissue. Although promising, rat CLiPs were demonstrated to exhibit chromosomal translocation under long-term culture. In addition, the YAC cocktail used to reprogram animal hepatocytes was not able to induce conversion in human mature hepatocytes. A year later, the same group was able to generate human CLiPs (hCLiPs) obtained from human infant hepatocytes using a similar strategy. The newly produced hCLiPs demonstrated significant repopulative capacity following transplantation into injured mice and upon redifferentiation in vitro exhibited enzymatic activities [191]. In addition, other groups have also reported different methods for in vitro generation of liver progenitors from human hepatocytes. Kim et al. [152] used a combination of similar small molecules (A83-01 and CHIR99021) and HGF to generate hepatic progenitors from human PHH. Those progenitors were highly proliferative and were capable of hepatic and biliary differentiation in vitro and in vivo. Contrary to those studies, Zhang et al. [156] observed that replacement of Wnt growth factor with CHIR99021 could not induce proliferation of hepatic cells in their 3D organoid system. More recently, Fu et al. [192] reported efficient expansion and differentiation of human hepatocyte−derived liver progenitor-like cells in vitro by activating NAD + dependent deacetylase signaling using specific transition and expansion medium formulations.

Conclusion

Recent advances in stem cell technologies and tissue engineering have resulted in the generation of prototype human liver tissue. These tissues display appropriate levels of liver function in vitro and in vivo, which evidences their potential. These advances, in combination with tissue engineering, microfabrication, and microfluidics, promise new opportunities to develop perfused human tissue for the clinic and the laboratory. Going forward, further development of 3D bioprinting techniques will help to refine tissue organization, vascularization, stability, and scale-up. We believe that advances in this scientific area will provide defined, renewable, and functional human liver tissue for translational medicine.

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Chapter 40

Hepatic tissue engineering

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Liver disease burden

Fatal liver disease accounts for ~2 million deaths annually worldwide and has steadily increasing rates over the years [1]. Liver failure can be divided into three major categories: (1) acute liver failure (ALF) that presents as a rapid loss of liver function in patients without preexisting liver disease, (2) chronic liver disease due to metabolic dysfunction, and (3) chronic liver failure accompanied by tissue remodeling and scarring.

ALF is a rare syndrome with an annual incidence of less than 10 cases per million people in the developed world. In the United States, ~2000 cases of ALF are diagnosed each year [2]. It commonly develops in healthy adults in their 30s. Patients with ALF usually present with abnormal liver biochemistry, coagulopathy, and encephalopathy. The causes vary geographically. Damage due to drug exposure (e.g., acetaminophen) is the most common cause in the West, while in large parts of the East, viruses (e.g., Hepatitis A and E) are the most prominent cause of ALF [3]. Clinically, ALF can be subdivided based on the period of time between the appearance of jaundice and onset of hepatic encephalopathy. Data from O'Grady et al. proposed the following classification: hyperacute for periods between 0 and 7 days, acute for periods between 7 and 28 days, and subacute for periods between 4 and 12 weeks [4]. In hyperacute cases the cause is usually acetaminophen toxicity or viral infection. Subacute cases that evolve slowly often result from idiosyncratic drug-induced liver injury (DILI). Even though patients with a subacute presentation have less coagulopathy and encephalopathy, paradoxically they have a consistently worse medical outcome than those with a more rapid onset of the disease [5].

Chronic liver disease develops on the background of a constant injurious insult, either resulting from a metabolic disorder or a number of etiologies that lead to widespread tissue remodeling and pathologic deposition of extracellular matrix (ECM). Inborn liver-based errors of metabolism are life-threatening conditions caused by genetic defects in single enzymes or transporters and lead to blockade of a specific metabolic pathway. While they can be accompanied by progressive fibrosis and cirrhosis, such as in the case of α1-antitrypsin ZZ deficiency, hemochromatosis, Wilson's disease, and hereditary tyrosinemia [6], the liver parenchyma often remains intact. Some examples of metabolic disorders with an intact parenchyma include hypercholesterolemia, Crigler–Najjar syndrome, ornithine transcarbamylase deficiency, organic acidurias, and hyperoxaluria [7]. In a biopsy the lack of parenchymal destruction often leads to a delayed diagnosis, exposing the patient to sequelae. In all cases of liver disease the lack of FDA-approved noninvasive biomarkers makes it challenging to diagnose and treat liver diseases.

Chronic liver disease occurs in the setting of nonalcoholic fatty liver disease (NAFLD). NAFLD is marked by hepatic steatosis and is related to the presence of metabolic syndrome in association with obesity, diabetes, and/or arterial hypertension [8]. A subset of NAFLD patients

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will develop signs of nonalcoholic steatohepatitis (NASH), a more severe condition associated with lobular inflammation and hepatocellular ballooning and can lead to fibrosis and cirrhosis [9]. In NAFLD the liver is unable to utilize carbohydrates and fatty acids properly, leading to toxic overaccumulation of lipid species. These metabolites induce cellular stress, injury, and death, which predispose the liver to sequelae such as cirrhosis and hepatocellular carcinoma [10].

In the United States the number of NAFLD cases is projected to expand from 83.1 million in 2015 (~26% of the population) to 100.9 million by 2030 (~28% of the population) [11]. An increasing percentage of these cases are projected to be classified as NASH, rising from 20% to 27% of adults with NAFLD during this interval [12]. While diagnosing NASH at an early stage remains a challenge, multiplexed protease-activated nanosensors have demonstrated utility in monitoring NASH progression and treatment response in a 3,5-diethylcarbonyl-1,4-dihydrocollidine model of fibrosis in mice. With further development, these noninvasive readouts can be used to diagnose disease.

Current state of liver therapies

In order to mitigate the clinical burden of liver disease, several therapeutic strategies have been undertaken (Fig. 40.1).

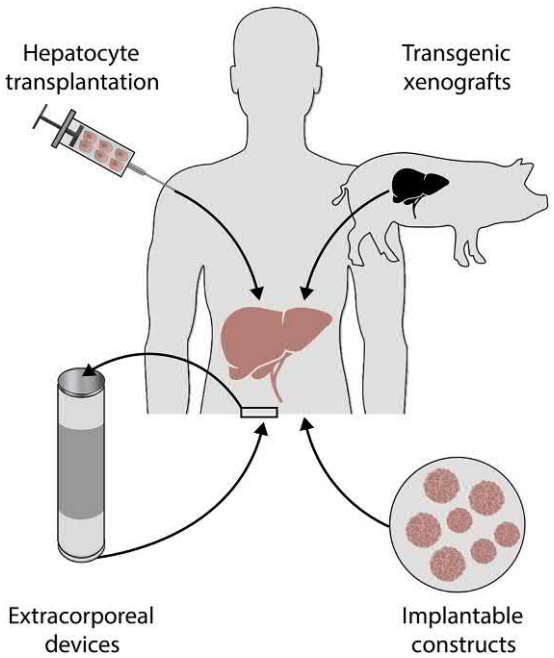

FIGURE 40.1 Cell-based therapies for liver disease. Extracorporeal devices perfuse patient's blood or plasma through bioreactors. Hepatocytes are transplanted directly or implanted onto scaffolds. Genetically modified large animals can be used for xenotranplantation.

Extracorporeal liver support devices

Liver failure is associated with abnormal accumulation of numerous endogenous substances such as bilirubin, ammonia, free fatty acids, and proinflammatory cytokines [13]. Extracorporeal liver support devices have been developed to detoxify the blood and plasma in order to bridge patients to liver transplantation (LT) or allow the native liver to recover from injury. Artificial liver (AL) devices use nonliving components for detoxification, such as membrane separation or sorbents, to selectively remove toxins but have limited clinical use because they do not replace the synthetic and metabolic roles of the liver [13]. Bioartificial liver (BAL) devices, on the other hand, contain a cell-housing bioreactor that aims to provide the detoxification and synthetic functions of the liver and are an ongoing topic of clinical investigation. Current versions are either based on hollow fiber cartridges [14–16] or on perfused three-dimensional (3D) matrices [17]. BALs, just like other hepatocyte-based therapies, face many challenges, such as the lack of readily available functional cell sources and the loss of cell viability and phenotype during the treatment process.

Biopharmaceuticals

In the setting of ALF, *N*-acetyl cysteine (NAC) is FDA-approved to reduce the extent of liver injury after acetaminophen overdose [18]. In the setting of chronic liver disease, however, most of the FDA-approved therapies are for hepatitis A, B, and C. A detailed listing can be found in Table 40.1. While a few treatments have shown moderate efficacy, there are currently no biopharmaceuticals that are approved for NAFLD, NASH, or cirrhosis. Glitazones, for example, upregulate adiponectin, an adipokine with antisteatogenic and insulin-sensitizing properties [19]. Vitamin E, an antioxidant, can prevent liver injury by blocking apoptotic pathways and protecting against oxidative stress [19]. Despite clinical studies of a large number of therapeutic candidates, no single agent or combination has shown improvement to liver-related morbidity and mortality in patients with NASH. Until a drug is FDA-approved for NASH indications, lifestyle modifications and optimizing metabolic risk factors are the best medical-treatment options for these patients.

Liver transplantation

The first attempt at human LT took place at the University of Colorado on March 1, 1963 but turned out to be unsuccessful [20]. Based on the pioneering work of Thomas Starzl, the first extended survival of a human recipient after LT was achieved on July 23, 1967 with a 19-month-old female patient with hepatocellular carcinoma who survived

TABLE 40.1 List of FDA-approved therapies for chronic liver diseases.

Drug name	Year approved	Indication(s)	Mechanism
Heplisav-B	2017	Hepatitis B	Combines hepatitis B surface antigen with a proprietary Toll-like receptor 9 agonist to enhance the immune response
Mavyret	2017	HCV genotype 1–6	Fixed-dose combination of glecaprevir, an HCV NS3/4A protease inhibitor, and pibrentasvir, an HCV NS5A inhibitor
Vosevi	2017	Hepatitis C	Fixed-dose combination of sofosbuvir, an HCV nucleotide analog NS5B polymerase inhibitor, velpatasvir, an HCV NS5A inhibitor, and voxilaprevir, an HCV NS3/4A protease inhibitor
Ocaliva	2016	Primary biliary cholangitis	FXR agonist
Zepatier	2016	HCV genotype 1 or 4	Fixed-dose combination product containing elbasvir, an HCV NS5A inhibitor, and grazoprevir, an HCV NS3/4A protease inhibitor
Cholbam	2015	Bile acid synthesis and peroxisomal disorders	Primary bile acid synthesized from cholesterol in the liver
Daklinza	2015	HCV genotype 3	Inhibitor of NS5A, a nonstructural protein encoded by HCV
Technivie	2015	HCV genotype 4	Fixed-dose combination of ombitasvir, an HCV NS5A inhibitor, paritaprevir, an HCV NS3/4A protease inhibitor, and ritonavir, a CYP3A inhibitor
Olysio	2013	Hepatitis C	Small molecule orally active inhibitor of the NS3/4A protease of HCV
Sovaldi	2013	Hepatitis C	Inhibitor of the HCV NS5B RNA-dependent RNA polymerase
Incivek	2011	HCV genotype 1	Inhibitor of the HCV NS3/4A serine protease
Victrelis	2011	HCV genotype 1	Inhibitor of the HCV NS3 serine protease
Viread	2008	Hepatitis B	Oral nucleotide analogue DNA polymerase inhibitor
Tyzeka	2006	Hepatitis B	Inhibitor of HBV DNA polymerase
Baraclude	2005	Chronic hepatitis B with evidence of active viral replication	Small-molecule guanosine nucleoside analog with selective activity against HBV polymerase
Hepsera	2002	Chronic hepatitis B with evidence of active viral replication	Inhibitor of HBV DNA polymerase
Pegasys	2002	Chronic hepatitis C with compensated liver disease	Binds to and activates human type 1 interferon receptors
Peg-intron	2001	Chronic hepatitis C	Binds to and activates human type 1 interferon receptors
Ribavirin	2001	Chronic hepatitis C	Synthetic nucleoside analog with antiviral activity
Twinrix	2001	Hepatitis A and B	Recombinant vaccine

FXR, Farnesoid X receptor; *HBV*, hepatitis B virus; *HCV*, hepatitis C virus; *NS3*, nonstructural protein 3.

13 months before succumbing to metastatic disease [21]. After the initial success of the surgery, advancements were made to improve donor organ quality, recipient selection, operative and perioperative management, immunosuppression and infectious complications. These advancements have made orthotopic LT the primary treatment for end-stage liver disease and certain cancers. These transplants have 1-year patient survival rates over 80% [22]. However, many challenges remain, including donor organ shortages, recipients with more advanced disease at transplant, a growing need for retransplantation, and adverse effects associated with long-term immunosuppression. To overcome a growing imbalance between the supply and demand of donor livers, transplant centers have developed strategies to expand the donor pool. These strategies include live donor LT [23], split-LT [24], and extended criteria for donor livers [25]. Despite all these efforts, the number of liver transplants has not increased in the last decade.

An alternative to human LT is xenotransplantation, though it has been clinically intractable due to concerns about immunological rejection and zoonotic pathogen transfer. With the advent of accessible genetic engineering technologies to circumvent the aforementioned challenges, the breeding efficiency of animals can be leveraged to mass-produce tissue for human organ transplants. Niu et al. applied CRISPR-Cas9 to inactivate all 62 copies of porcine endogenous retroviruses, thus paving the way for pig-to-human transplants [26]. Relatedly, Längin et al. genetically engineered porcine heart xenografts and demonstrated long-term pig-to-baboon orthotopic transplantation [27].

Hepatocyte transplantation

Given the several drawbacks of LTs, alternative strategies have been pursued. A potential alternative to LT is allogeneic hepatocyte transplantation (HT). Transplanted cells can provide the missing or impaired hepatic function once engrafted. Given their synthetic and metabolic capabilities, mature hepatocytes are the primary candidates for liver cell transplantations. HT offers several advantages over LT. It is less invasive and can be performed repeatedly to meet metabolic requirements. Furthermore, multiple patients can be treated with a single dissociated donor tissue, and harvested cells can be cryopreserved for later use on an as-needed basis.

The first experimental attempt of HT was done in 1976 to treat an animal model for Crigler–Najjar syndrome type I [28]. Along with other observations, it led to the first transplant of autologous hepatocytes in 10 patients with liver cirrhosis in 1992 in Japan [29]. Since then, reports have been published on more than 100 patients with liver disease treated by HT worldwide [30]. Human HT has resulted in partial correction of a number of liver diseases, including urea cycle disorders [31], factor VII deficiency [32], glycogen storage disease type 1 [33], infantile Refsum disease [34], phenylketonuria [35], severe infantile oxalosis [36], and ALF [37]. HT faces several limitations: limited supply of high-quality mature hepatocytes, freeze–thaw damage due to cryopreservation, poor cellular engraftment (estimated to be from 0.1% to 0.3% of host liver mass in mice after infusion of 3% − 5% of the total recipient liver cells) [30], and allogeneic rejection.

Clinically, the most widely used administration route for HT is through the portal vein or one of its branches. Hepatocytes traverse the sinusoidal vasculature and create transient occlusions. The occlusions lead to vascular permeabilization which allows transplanted cells to reach the liver parenchyma [35]. The number of cells that are injected intraportally and subsequently engraft is a function of portal pressure and liver architecture. Thus other administration routes have been explored for patients with cirrhosis who have high portal pressures due to fibrosis.

In animal studies, hepatocytes transplanted into the spleen proliferate for extended periods of time and display normal hepatic function. The spleen has been shown to be well-suited for hepatocyte engraftment because it functions as a vascular filter and provides an immediate blood supply [30]. The peritoneal cavity represents an attractive administration route as it is easily accessible and can house a large number of cells. Due to cell number requirements associated with metabolic compensation, it has been used in patients with ALF [38]. As an alternative to the portal vein, spleen and peritoneum, the lymph node (LN) has also been shown to demonstrate engraftment of donor hepatocytes [39,40]. While this strategy has not been utilized in the clinic yet, the preclinical data is promising.

Current clinical trials

Several pathways have been implicated in the biology and pathogenesis of NAFLD development: insulin resistance, lipotoxicity, oxidative stress, altered immune/cytokine/mitochondrial functioning, and apoptosis. New therapeutic modalities are being developed to target many of these pathways. For a detailed overview of NAFLD-targeted drugs that are currently in the clinical trial pipeline, please refer to Younossi et al. [41].

In vitro models

To build high-fidelity cellular models and therapies, components of the native liver microenvironment must be incorporated (Fig. 40.2). The liver's highly organized structure is key to its role as a complex tissue supporting myriad synthetic and metabolic functions. In addition to hepatocytes the main parenchyma of the liver, there are several nonparenchymal cell types such as liver sinusoidal endothelial cells, Kupffer cells, cholangiocytes, and stellate cells. In each lobule of the liver an array of parallel hepatocyte cords are sandwiched between the sinusoid, carrying circulating blood, and the bile duct, carrying hepatocyte-secreted bile acids. Notably, this arrangement dictates a unique set of architecturally driven cell–cell and cell–matrix cues, which gives rise to liver-specific phenotypes. Gradients of physicochemical stimuli along the sinusoid drive zonal phenotypes with disparate metabolic and synthetic functional profiles [42]. Interrupting the natural order of cell arrangement in the liver is directly connected with diseases discussed in the "Liver disease burden" section. In this chapter, we will primarily focus on human platforms, which are biologically distinct from animal-derived cell models of the liver that are reviewed more in depth elsewhere [43].

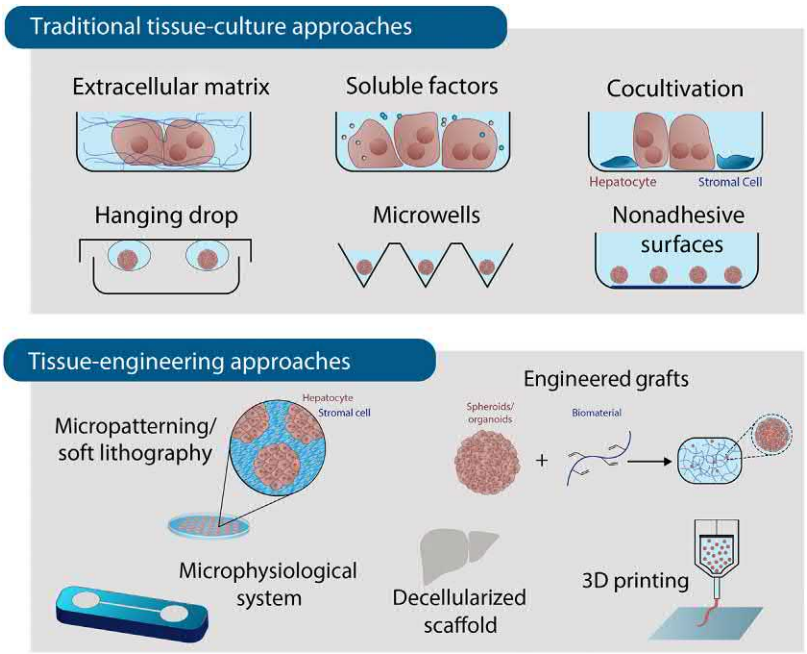

FIGURE 40.2 Advances in hepatic tissue engineering. Traditional tissue culture approaches such as the addition of extracellular matrix, soluble factors, cocultivation with supporting cell types, hanging drop, microwell molding, and nonadhesive surfaces have enabled the early study of hepatocyte phenotype in vitro in both 2D and 3D cultures. The advent of technologies from disciplines such as chemical engineering and electrical engineering has led to a new level of control for hepatic tissue cultures, such as micropatterning to template cell interactions, microphysiological systems to study the impact of bioactive perfusate, polymeric biomaterials for constructing 3D cell-laden grafts, perfusion technologies for decellularization/recellularization strategies, and 3D printing for scalable engineering of cellular grafts. *2D*, Two-dimensional; *3D*, three-dimensional.

Two-dimensional liver culture

Hepatocytes are responsible for more than 500 metabolic and synthetic functions of the human body, often categorized broadly as protein synthesis and secretion, detoxification, bile synthesis, and nitrogen metabolism. Primary hepatocytes quickly lose their phenotype and function after a few days in traditional monolayer culture and require a collagen-coated surface for adherence and survival [6]. In contrast, when primary human hepatocytes (PHHs) are cultured between two layers of collagen gel (i.e., sandwich culture configuration), they retain viability, polarity and many axes of relevant metabolic and synthetic function [44,45]. Guguen-Guillouzo et al. found that a random coculture with a liver epithelial cell line was sufficient to support hepatic albumin secretion, suggesting the importance of heterotypic cell interactions for long-term ex vivo culture [46]. Bale et al. demonstrated that other nonparenchymal liver cells can better recapitulate hepatic response to inflammatory stimuli, through higher order intercellular interactions captured in a multicellular platform [47]. It was later discovered that a micropatterned architecture consisting of hepatocyte-filled islands surrounded by a nonbiomimetic cell type and mouse fibroblasts can also stabilize hepatocytes, suggesting the existence of conserved coculture signals across species. These micropatterned cocultures (MPCCs) enable the study of DILI and hepatotropic pathogen infection for several weeks in vitro [48–50]. Furthermore, Davidson et al. added hepatic stellate cells to the traditional MPCC to create an in vitro model of NASH [51].

Despite the utility of two-dimensional (2D) hepatic cultures in screening assays, a wealth of literature suggests that they are dissimilar to hepatocytes in vivo. Specifically, 2D formats, even with overlaid collagen matrix, are more flattened than their native cuboidal architecture. On a subcellular level, this translates to major differences in cytoarchitecture, which is linked to aberrant polarization and nonphysiological behavior [52,53]. Griffith and Swartz have described the improved presentation of relevant biochemical and mechanical cues in 3D cultures, typically cell-laden hydrogels, compared to traditional 2D cultures [54].

Three-dimensional liver constructs

Commonly, 3D hepatic cultures consist of primary cell or induced pluripotent stem cell (iPSC)-derived spheroid and organoid cultures, which are typically embedded in ECM-based hydrogels [55]. Spheroids and organoids can be manufactured using a variety of techniques, such as microwell mold-based technologies, which offer a high degree of composition and size control but are difficult to scale. Spinning flasks and bioreactors can produce large populations of spheroids, though they are typically nonuniform in size and function. Bell et al. showed that primary human hepatic spheroids fabricated and cultured in microwell plates serve as useful models of hepatotoxicity and multiple liver pathologies [56].

Toward transplantable cell therapies, Stevens et al. used microwell molds to create 3D hepatic spheroid

cocultures of PHHs and fibroblasts, which can be embedded in agarose, fibrin, or polyethylene glycol hydrogel scaffolds. The resulting tissue constructs support hepatic function in vitro and in vivo after ectopic transplantation into the peritoneal cavity [57]. Furthermore, Stevens et al. demonstrated that implanting a tissue seed, consisting of hepatic aggregates and vascular cords, into an FRNG [fumarylacetoacetate hydrolase-deficient ($Fah^{-/-}$), recombinase activating gene-deficient ($Rag1^{-/-}$), nonobese diabetes (NOD), and interleukin-2 receptor γ chain-deficient (Il2rγ-null)] mouse model of hereditary tyrosinemia leads to a 50-fold expansion in serum human albumin and formation of perfusable vessels after 80 days of cycled exposure to regenerative stimuli [58]. Relatedly, Takebe et al. constructed liver buds consisting of iPSC-derived hepatocyte-like cells (HLCs), mesenchymal stem cells, and human umbilical vein endothelial cells; mesenteric transplantation of these human liver buds resulted in vascularization and rescued a lethal TK-NOG mouse model of liver injury [59].

To create clinically viable engineered liver constructs, cell sourcing (discussed further in the "Cell sourcing" section), and clinical-scale manufacturing of organoids and spheroids must be addressed. Toward this end, Takebe et al. constructed a large-scale liver bud microwell culture platform, enabling the formation of 10^8 liver buds [60].

Physiological microfluidic models of liver

Despite improvements to in vitro liver platforms as model systems and implants, static cultures lack physiologically relevant dynamic components. The native liver's dynamic physiology arises from blood circulation and multiorgan cross talk. Thus to improve biological fidelity, many have attempted to create microfluidic models of the liver [61]. By leveraging techniques from the semiconductor manufacturing industry such as soft lithography, groups have fabricated microphysiological systems with preformed channels to allow for perfusion of nutrients and to aid in waste removal [62–67]. These so-called liver-on-chip platforms allow for the study of biochemical and mechanical cues such as growth factor gradients and shear stress. Lee et al. demonstrated that the presence of human stellate cells and application of shear via flow enabled the formation of stable hepatic spheroids on chip [68]. Furthermore, by linking microfluidic channels between multiple tissue models, multiorgan phenomena such as drug metabolite toxicity and disease progression can be captured in vitro, which is not possible in traditional cultures [69–71].

Controlling three-dimensional architecture and cellular organization

Another approach to improving the functionality of tissue-engineered constructs is to more closely mimic in vivo microarchitecture by generating scaffolds with a highly defined material and cellular architecture, which would provide better control over the 3D environment at the microscale.

A range of rapid prototyping and patterning strategies have been developed for polymers using multiple modes of assembly, including fabrication using heat, light, adhesives, or molding, and these techniques have been extensively reviewed elsewhere [72]. For example, 3D printing with adhesives combined with particulate leaching has been utilized to generate porous poly(lactic-co-glycolic acid (PLGA) scaffolds for hepatocyte attachment [73], and microstructured ceramic [74] and silicon scaffolds [75,76] have been proposed as platforms for hepatocyte culture. Furthermore, molding and microsyringe deposition have been demonstrated to be robust methods for fabricating specified 3D PLGA structures toward the integration into implantable systems [77].

Microfabrication techniques have similarly been employed for the generation of patterned cellular hydrogel constructs. For instance, microfluidic molding has been used to form biological gels containing cells into various patterns [78]. In addition, syringe deposition in conjunction with micropositioning was recently illustrated as a means to generate patterned gelatin hydrogels containing hepatocytes [79]. Patterning of synthetic hydrogel systems has also recently been explored. Specifically, the photopolymerization property of poly(ethylene glycol) (PEG) hydrogels enables the adaptation of photolithographic techniques to generate patterned hydrogel networks. In this process, patterned masks printed on transparencies act to localize the ultraviolet exposure of the prepolymer solution and, thus, dictate the structure of the resultant hydrogel. The major advantages of photolithography-based techniques for patterning of hydrogel structures are its simplicity and flexibility. Photopatterning has been employed to surface pattern biological factors [80], produce hydrogel structures with a range of sizes and shapes [81,82], as well as build multilayer cellular networks [83,84]. Consequently, hydrogel photopatterning technology is ideally suited for the regulation of scaffold architecture at the multiple length scales required for implantable hepatocellular constructs. As a demonstration of these capabilities, photopatterning of PEG hydrogels was utilized to generate hepatocyte and fibroblast coculture hydrogels with a defined 3D branched network, resulting in improved hepatocyte viability and functions under perfusion [85]. More recently, a "bottom-up" approach for fabricating multicellular tissue constructs utilizing DNA-templated assembly of 3D cell-laden hydrogel microtissues demonstrates robust patterning of cellular hydrogel constructs containing numerous cell types [86]. Also, the additional combination of photopatterning with dielectrophoresis-mediated cell patterning enabled the construction of hepatocellular hydrogel

structures organized at the cellular scale. Overall, the ability to dictate scaffold architecture coupled with other advances in scaffold material properties, chemistries, and the incorporation of bioactive elements (discussed further in the "Extracellular matrix for cell therapies" section) will serve as the foundation for the future development of improved tissue-engineered liver constructs that can be customized spatially, physically, and chemically.

In vivo models

While there have been impressive advances in cell culture models of the human liver, experimental animal models still play an important role in the effort to engineer liver therapies. Commonly performed surgeries such as bile duct ligation and partial hepatectomy are experimentally tractable models of acute liver injury, yet they are of little clinical relevance. Drug-induced (e.g., carbon tetrachloride, acetaminophen, or thioacetamide) hepatotoxicity to induce necrotic lesions is more recapitulative of human pathophysiology, but the phenotype is difficult to reproduce [87]. In addition, modeling chronic liver injury in animal models is problematic because they tend to rapidly correct severe hepatic damage after a few days, which is not representative of human disease progression and resolution [88].

In order to model a human-like context for liver injuries, it is necessary to develop improved, controlled models of human liver injury. Such animal models can be useful for the evaluation of human liver biology and the preclinical performance of candidate therapies and drugs [89,90]. To accomplish this, human hepatocytes can be transplanted orthotopically in immunocompromised mice with no liver injury via the injection of a cell solution and are useful for modeling human-specific drug metabolism, liver injury, and hepatotropic infections. However, on average, HT exhibits poor levels of engraftment ($\sim$10%–30%) [91].

Transplanted hepatocytes have the ability to expand preferentially if the host is compromised by injury or genetic modification. The first genetically engineered mouse model to demonstrate this was the Alb-uPA mouse that carries a uroplasminogen activator under an albumin promoter, causing liver injury and failure [92]. Aiming to improve on the Alb-uPA system, a transgenic model of hereditary tyrosinemia I was developed, in which a genetic knockout of FAH leads to the hepatotoxic accumulation of fumarylacetoacetate [93]. FAH knockout mouse injury initiation and duration can be controlled through the administration of a small molecule drug [2-(2-nitro-3-trifluoro-methylbenzoyl)-1,3-cyclohexanedione] in the drinking water. Another inducible model called TK-NOG, which expresses thymidine kinase under an albumin promoter, causes hepatocyte ablation following activation by ganciclovir treatment [94]. In the AFC8 injury model, induction by the small molecule AP20187 drives caspase-8-initiated apoptosis of hepatocytes modified to express FK506 under an albumin promoter [95]. For all of the above models, engraftment rates surpassing 70% have been observed.

A classical study in parabiotic rats in the 1960s revealed that hepatic injury results in the expression of systemic, soluble signals that have the potential to drive liver regeneration [96]. Despite decades of research, the complex signaling cascade driving liver regeneration is still not well understood but has found utility in ectopic humanized mouse models. Chronic liver injury often presents with high portal pressures, which can reduce engraftment levels during an orthotopic transplantation. Thus transplantation to ectopic sites is clinically attractive. Ectopic grafts that anastomose to the host vasculature can interact with regenerative stimuli from the host liver, causing expansion and proliferation of the transplanted human hepatocytes. Initially, ectopic transplantation was demonstrated in the LN [40] and spleen [97], and later in the subcutaneous space and mesenteric fat pad, both of which offer ease of accessibility for manipulation and noninvasive imaging [58,59,98].

Taken together, the range of liver injury animal models are an essential tool for studying various perturbations to normal liver biology and building implantable tissue constructs to address acute and chronic liver failure. The field is just beginning to uncover mechanisms that control liver regeneration in various disease and injury contexts. The discovery of new soluble regenerative signals will be central to advancing therapies that have the potential to improve the supply of donor tissue.

Cell sourcing

Cell number requirements

The development of cell-based therapies poses myriad challenges, partially stemming from the scale of the liver. An adult human liver is estimated to possess 241 billion hepatocytes, 24 billion stellate cells, and 96 billion Kupffer cells [99]. Sourcing such enormous cell numbers using current technologies is not feasible.

However, many human HT studies suggest that clinical intervention is possible with a fewer number of cells and offer critical insights to help us determine minimum cell numbers. In a review, Fisher and Strom cataloged 78 different human HT studies, detailing both the input cell number and a qualitative description of functionality [30]. Correlating these, we can broadly surmise that to correct inborn errors of metabolism, at least 1–10 billion hepatocytes are needed. However, for ALF, that number grows to 5–20 billion cells. For liver cirrhosis, HTs have largely been unsuccessful (discussed further in the "Hepatocyte transplantation" section); therefore it is unclear what the

cellular requirements for cirrhosis are. While injection of hepatocytes is not the same as implantation within a scaffold, these studies serve as useful inputs into more complex physiological models.

In order to get us closer to these numbers, many different cell sources have been explored.

Immortalized cell lines

Immortalized hepatocyte cell lines can be derived from liver tumor tissue or directly from primary hepatocytes in vitro. The prominent lines utilized today are HepG2, derived from hepatocellular carcinoma; HepaRG [100], a human bipotential progenitor cell line; C3A, derived from HepG2s; and Huh7, derived from liver tumor [101]. Several other fetal and adult hepatic cell lines have also been established, typically using a combination of viral oncogenes and the human telomerase reverse transcriptase protein [102]. However, these cell lines lack the full functional capacity of primary adult hepatocytes and there is a risk that oncogenic factors could be transmitted to the patient, limiting their use as a cell source for transplantation therapies.

Primary cells

Unlike immortalized lines, PHHs can provide a whole host of human liver-specific function. PHHs, however, are limited in supply, and their phenotype is difficult to maintain in vitro. Many methods have been developed for maintaining long-term functionality of hepatocytes through the use of a variety of configurations and biomaterial constructs, which are further discussed in the "In vitro models" section. Due to limitations in the supply of mature human hepatocytes, many groups have attempted to promote the expansion and proliferation of PHHs in vitro. Peng et al. have shown that TNFα promotes the expansion of hepatocytes in 3D cultures and enables serial passaging and long-term culture for more than 6 months [103]. In a similarly notable study, Hu et al. identified an optimal cell culture cocktail consisting of B27 supplement (without vitamin A), R-spondin, CHIR99021 (a Wnt agonist), NAC, nicotinamide, gastrin, epidermal growth factor (EGF), TGFα, fibroblast growth factor (FGF)7, FGF10, HGF, a TGFβ inhibitor (A83-01), and ROCK inhibitor that led to long-term 3D organoid culture of PHHs [104].

Fetal and adult progenitors

Given their ability to differentiate into diverse lineages both in vitro and in vivo, iPSC and human embryonic stem cell cultures can also be utilized to generate HLCs. Various differentiation protocols have been applied to these cultures to yield cell populations that exhibit some phenotypic and functional characteristics of hepatocytes [62,105–108]. These populations are termed HLCs because of their expression of fetal proteins and fetal-like cytochrome P450 profiles [109]. While they are distinct from mature adult hepatocytes, HLCs can still serve as a potential cell source in very specific contexts.

In addition to pluripotent cells, bipotential progenitor cells can also serve as a source for hepatocytes. Huch et al. delineated conditions that allow for long-term expansion of adult bile duct-derived EpCAM + bipotential progenitor cells from the human liver [110]. The expanded cell population attained using their protocol is stable at the chromosomal level and can be converted into functional HLCs in vitro and in vivo [110].

Reprogrammed hepatocytes

HLCs can also be generated using direct reprogramming of mature cell types. For example, several groups have demonstrated the feasibility of reprogramming fibroblasts into HLCs without a pluripotent intermediate [111–113]. Cheng et al. demonstrated that a combination of nuclear factors can stimulate the conversion of hepatoma cells to HLCs [101]. These findings raise the future possibility of deriving human HLCs directly from another adult cell type.

Extracellular matrix for cell therapies

The ECM of the liver provides a structural scaffold with bioactive cues that modulate hepatic function and promote vascularization. Collagen and fibronectin are the major structural components of the liver ECM. Along with other nonstructural proteins, these components participate in integrin-mediated signaling between cells and their surrounding matrix. Hepatocytes are sensitive to their ECM, and it has been demonstrated that the presence of abnormal amounts and/or types of ECM components correlates with the onset and progression of liver fibrosis [114].

ECM scaffolds for hepatic tissue engineering are useful for constructing 3D tissue models and as a delivery vehicle for implants. Polymeric biomaterial hydrogels gained popularity as an engineering tool for recapitulating a physiologically relevant 3D tissue niche. Aside from creating a permissive environment for hepatocyte survival and growth, ECM scaffolds for hepatic tissue engineering also enable the formation of biliary and vascular networks that will be further discussed in the "Vascular and biliary tissue engineering" section. Broadly speaking, ECM scaffolds can be constructed using synthetic and/or naturally derived polymers.

Natural scaffold chemistry and modifications

A wide range of natural biomaterial polymers spanning polysaccharides (e.g., dextran and chitosan), peptides (e.g., collagen and fibrin), decellularized ECM (dECM), and composites of these have been employed as hepatic tissue−engineering scaffolds [55,57,58,115−121]. The advantages of biologically derived materials include their biocompatibility; naturally occurring cell adhesive moieties; and, in the case of decellularization, native architectural presentation of ECM molecules. However, naturally derived biomaterials have several barriers to use in the clinic, primarily due to lot-to-lot variability and xenogeneic origin.

The choice of material determines the physicochemical and biological properties of the scaffold. For example, early efforts in developing implantable hepatic constructs utilized collagen-coated dextran microcarriers that enabled hepatocyte attachment since hepatocytes are known to be anchorage-dependent cells. The intraperitoneal transplantation of these hepatocyte-attached microcarriers resulted in successful replacement of liver functions in two different rodent models of genetic liver disorders [122]. Subsequently, collagen-coated or peptide-modified cellulose [120,123], gelatin [124], and gelatin−chitosan composite [125] microcarrier chemistries have also been explored for their capacity to promote hepatocyte attachment. On the other hand, materials that are poorly cell adhesive such as alginate [115] have been exploited for their utility in promoting hepatocyte−hepatocyte aggregation (i.e., spheroid formation) and phenotypic stabilization within these scaffolds. Collectively, the size of engineered tissues created by these approaches is limited by oxygen and nutrient diffusion to only a few hundred microns in thickness.

To address this constraint, recent work has sought to use decellularized whole organ tissue as a matrix for liver tissue engineering. The decellularization process utilizes perfusion-based technologies to remove cells from donor tissues but preserve the structural and functional characteristics of the native underlying tissue. Recent advances in decellularization protocols have yielded scaffolds with native liver ECM composition, growth factor presentation, vascular structure, and biliary network architecture [126−128]. To date, seeding protocols have achieved up to 95% efficiency of recellularization with relevant cell populations (e.g., hepatocytes, vascular cells and bipotent hepatic progenitors); resulting recellularized grafts exhibited liver-specific function, and survival after transplantation in rodents [121,126,129]. Furthermore, cell-laden, xeno-derived dECM scaffolds are compatible with immunocompetent animal models [128]. However, given the shortage of donor tissue, the wide use of dECM scaffolds is unlikely.

Synthetic scaffold chemistry

In contrast to biologically derived material systems, synthetic materials enable precisely customized architecture (porosity and topography), mechanical and chemical properties, and degradation modality and kinetics, which are known to drive cell behavior. Synthetic materials that have been explored for liver tissue engineering include poly(L-lactic acid) (PLLA), PLGA, poly(ε-caprolactone), and PEG [85,98,130−135]. Polyesters such as PLLA and PLGA are the most common synthetic polymers utilized in the generation of porous tissue-engineering constructs. These materials are biocompatible, biodegradable, and have been used as scaffolds for HT [132,136]. A key advantage of PLGA is the potential to finely tune its degradation time due to differences in susceptibility to hydrolysis of the ester groups of its monomeric components (lactic acid and glycolic acid). However, the accumulation of hydrolytic degradation products has been shown to produce an acidic environment within the scaffold which initiates peptide degradation and stimulates inflammation, which may affect hepatocyte function [137]. Consequently, as alternatives to macroporous scaffold systems, approaches aimed at the efficient and homogeneous encapsulation of hepatocytes within a fully 3D structure have been explored. In particular, hydrogels that exhibit high water content and thus similar mechanical properties to tissues are widely utilized for various tissue-engineering applications, including hepatocellular platforms. Synthetic, PEG-based hydrogels have been increasingly utilized in liver tissue-engineering applications due to their high water content, hydrophilicity, resistance to protein adsorption, biocompatibility, ease of chemical modification, and the ability to be polymerized in the presence of cells, thereby enabling the fabrication of 3D networks with uniform cellular distribution [138]. PEG-based hydrogels have been used for the encapsulation of diverse cell types, including immortalized and primary hepatocytes and hepatoblastoma cell lines [85,98,135]. The encapsulation of primary hepatocytes requires distinct material modifications [e.g., 10% w/v PEG hydrogel, inclusion of RGD adhesive motifs] as detailed below, as well as, analogous to 2D coculture systems, the inclusion of nonparenchymal supporting cell types such as fibroblasts and endothelial cells [135].

Modifications in scaffold chemistry

The relatively inert nature of synthetic scaffolds allows for the controlled incorporation of chemical/polymer moieties or biologically active factors to regulate different aspects of cellular function. Chemical modifications such as oxygen plasma treatment or alkali hydrolysis of PLGA [139,140], or the incorporation of polymers such as

poly(vinyl alcohol) or poly(*N*-*p*-vinylbenzyl-4-*O*-β-D-galactopyranosyl-D-glucoamide) (PVLA) into PLGA or PLLA scaffolds [132,141,142] have improved hepatocyte adhesion by modulating the hydrophilicity of the scaffold surface [143]. Biological factors may include whole biomolecules or short bioactive peptides. Whole biomolecules are typically incorporated by nonspecific adsorption of ECM molecules such as collagen, laminin, or fibronectin [139,144]; covalent conjugation of sugar molecules such as heparin [145,146], galactose [131,147], lactose [145] or fructose [148]; or growth factors such as EGF [149]. Alternatively, short bioadhesive peptides that interact with cell surface integrin receptors have been extensively utilized to promote hepatocyte attachment in synthetic scaffolds. For example, conjugation of the RGD peptide to PLLA has been shown to enhance hepatocyte attachment [150], whereas RGD modification significantly improved the stability of long-term hepatocyte function in PEG hydrogels [98,135]. The additional incorporation of adhesive peptides that bind other integrins may serve as a way to further modulate and enhance hepatocyte function within synthetic polymer substrates. Moreover, Stevens et al. demonstrated that integration of matrix metalloproteinase-sensitive peptide sequences into hydrogel networks as degradable linkages has been shown to enable cell-mediated remodeling of the hydrogel [151].

The capacity to modify biomaterial scaffold chemistry through the introduction of biologically active factors will likely enable the finely tuned regulation of cell function and interactions with host tissues important for implantable systems.

Porosity

A common feature of many implantable tissue-engineering approaches is the use of porous scaffolds that provide mechanical support, often in conjunction with cues for growth and morphogenesis. Collagen sponges, various alginate and chitosan composites, and PLGA are the most commonly used porous scaffolds for hepatocyte culture and are generally synthesized using freeze-dry or gas-foaming techniques. Pore size has been found to regulate cell spreading and cell−cell interactions, both of which can influence hepatocyte functions [116], and may also influence angiogenesis and tissue ingrowth [152]. Porous, acellular scaffolds are normally seeded using gravity or centrifugal forces, capillary action, convective flow, or through cellular recruitment with chemokines, but hepatocyte seeding is generally heterogeneous in these scaffolds [153,154].

Vascular and biliary tissue engineering

Beyond compatibility with hepatic cell types, scaffolds should also be conducive to vasculature formation. Relying on vascularization by the host is not sufficient for large tissue constructs required for the clinic, because cells that are not near capillary structures ($>150-200\ \mu m$) are at a risk for necrosis after a matter of hours due to a lack of oxygen, nutrient availability, and waste transport. In this section, we discuss composite approaches toward building scalable, vascularized constructs.

Vascular engineering

Approaches to engineering vessels can generally be categorized as bottom-up induction of vascular assembly and top-down fabrication of vascular conduits [155]. Bottom-up vascular engineering approaches are built upon the idea of neovascularization, or new vessel formation. Vessel formation can occur by angiogenic sprouting, the formation of vessels branching off of an existing blood vessel, or vasculogenesis, the self-assembly of single endothelial cells or progenitor cells into lumenized vessels. Despite the ability of single vascular cells to coalesce to enable self-assembly, vasculogenesis is accelerated by coculture with supporting stromal cells, such as fibroblasts, mesenchymal stem cells, and pericytes [155−157]. Studies exploring angiogenesis and sprouting events suggest that chemical gradients, fluid-driven shear stress, and a hypoxia are key players in vessel formation [158−161].

Top-down fabrication approaches dictate geometry and architecture, rather than driving self-assembly. Polymer molding using microetched silicon has been shown to generate extensive channel networks with capillary dimensions, though it is not amenable to high-throughput manufacturing [162]. While direct printing of cells can be cytotoxic, 3D printing has become a popular approach for fabricating hollow channels that enable vascular cell seeding. The challenge lies in using this approach to build patent capillary beds, which are $5-10\ \mu m$ in diameter. While two-photon polymerization has an impressively high feature size resolution at 100 nm, the trade-off between build volume (i.e., building constructs large enough for clinical impact), build speed (i.e., impacting manufacturability), and printer resolution renders it inappropriate for most applications in tissue engineering. Alternate printers using direct-ink writing (DIW) of viscoelastic materials have emerged as a powerful tool for the fabrication of patterned hydrogel constructs. DIW can achieve minimum feature size resolutions from 1 to 250 μm, depending on the ink "building block" size [163]. However, DIW printing requires yield-stress fluid inks with restrictive viscosities (10^2-10^6 mPa s), such that they fluidize under stress but regain the original shear elastic modulus after printing [163]. Kolesky et al. demonstrated that DIW and fugitive inks were useful for multimaterial printing as well as

construction of thick (>1 cm), vascularized tissues [164,165]. Miller et al. demonstrate the use of thermal microextrusion approach to create sacrificial sugar glass lattices that can be embedded in various cell-laden biomaterials, evacuated and subsequently lined with vascular cells [166].

Taken together, self-assembly driven formation of a capillary bed can be combined with printing of larger vessels to enable the fabrication of a fully vascularized tissue construct. Song et al. used 3D printing to create curved vascular channels using a fugitive ink [159]. The vascular cells lining the channel were able to degrade the surrounding hydrogel matrix and undergo sprouting when exposed to angiogenic factors. They further demonstrated that the curvature and complexity of printed vasculature impacted the extent of sprouting, which will be an important consideration for vessel patterning in future clinical applications [159].

Host integration

For vascularized constructs to survive after implantation the engineered vessels must anastomose with the host vasculature. To date, the exact mechanism that drives integration with the host is not well understood [155]. A number of studies have elucidated the contribution of cytokines important in angiogenesis and recruitment of host vasculature in implant constructs, such as vascular EGF (VEGF) [136], basic FGF [167], and VEGF in combination with platelet-derived growth factor [168]. Furthermore, preimplantation of VEGF releasing alginate scaffolds prior to hepatocyte seeding was demonstrated to enhance capillary density and improve engraftment [169]. In addition, while building an AL tissue construct, Stevens et al. demonstrated that the coimplantation of parallel endothelial cell cords with PHH and fibroblast spheroids led to better hepatic performance and survival when compared to an implant with nonpatterned endothelial cells, suggesting that there may be an optimal templated endothelial geometry that enables vascularization and host integration in vivo [58]. Surgical anastomosis poses an alternative to biologically driven anastomosis, though this approach requires invasive surgery and access to suture-able vessels both in the graft and in the host. Strategies to incorporate vasculature into engineered constructs include the microfabrication of vascular units with accompanying surgical anastomosis during implantation [75,170].

In addition to interactions with the vasculature, integration with other aspects of host tissue will constitute important future design parameters. For instance, incorporation of hydrolytic or protease-sensitive domains into hepatocellular hydrogel constructs could enable the degradation of these systems following implantation [151]. Of note, liver regeneration proceeds in conjunction with a distinctive array of remodeling processes such as protease expression and ECM deposition. Interfacing with these features could provide a mechanism for the efficient integration of implantable constructs. Similarly to whole liver or cell transplantation, the host immune response following the transplant of tissue-engineered constructs is also a major consideration. Immunosuppressive treatments will likely play an important role in initial therapies, although stem cell–based approaches hold the promise of implantable systems with autologous cells. Furthermore, harnessing the liver's unique ability to induce antigen-specific tolerance [171] could potentially represent another means for improving the acceptance of engineered grafts.

Biliary network engineering

Importantly, future iterations of hepatic grafts should include a biliary system, which is responsible for excretory functions. In a similar vein, we envision that a combination of "top-down" manufacturing, such as the aforementioned technologies for generating patent vascular conduits, and "bottom-up" approaches, which could involve leveraging biological phenomena that drive biliary morphogenesis, will be useful for building a biliary network.

The biliary tree is a complex, 3D network of tubular conduits of various sizes and properties. The liver contains an intrahepatic compartment that is lined by biliary epithelial cells, termed cholangiocytes, that aid in the modification and removal of hepatocyte-secreted bile. Even though the blood vessel–fabrication approaches delineated above have not yet been applied to engineering bile networks for implantable liver constructs, advancements in cholangiocyte sourcing methods have made it feasible. Sampaziotis et al. identified a protocol for directed differentiation of human iPSCs into cholangiocyte-like cells [172]. In 2017 the same group provided the first proof-of-concept study to reconstruct the gallbladder wall and repair the biliary epithelium using human primary cholangiocytes expanded in vitro [173]. Furthermore, current studies are focused on the development of in vitro models that exhibits biliary morphogenesis and recapitulates appropriate polarization and bile canaliculi organization [174–176], as well as platforms for the engineering of artificial bile duct structures [177,178].

Conclusion and outlook

Traditionally, hepatic tissue–engineering research has focused on designing the microenvironment to support a stable hepatic phenotype. As concomitant advances in pluripotent cell research and polymer chemistry have been actualized, new cell sources and extracellular

matrices have been added to the pipeline. This interdisciplinary synergy has been the driving force behind the development of tissue-engineered grafts with long-term survival and growth. In order to inch closer to the regenerative medicine north star of an ex vivo engineered graft that can serve as a replacement for the native organ, there are several areas to consider for improvement: (1) vascularization of thick, dense grafts through a combination of self-assembly and bioprinting; (2) engineering of the hepatic graft to prevent immune rejection in allogeneic and xenogeneic settings; (3) improved understanding of metabolic and cellular requirements of various liver diseases; (4) development of scalable, renewable cell sources that do not compromise the functional capabilities of cells; (5) leveraging of animal injury models as bioreactors for cell sourcing; and (6) upscaling of grafts in a manner that is compatible with FDA's Good Manufacturing Practice standards.

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Part Twelve

Hematopoietic system

Chapter 41

Hematopoietic stem cells

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Introduction

Transplantation of hematopoietic stem cells (HSCs) shows promising prospect for broad-spectrum hematological disorder therapy, owing to their potential to generate the entire hematopoietic lineages [1,2]. However, the limited number of HSCs in either bone marrow (BM) or umbilical cord blood (UCB) limits their widespread use [3]. Hence, there is great interest in developing methods for ex vivo expansion and thus self-renewal of HSCs [4,5]. Strategies based on nanomaterials or nanotechnologies seem hopeful to expand HSC numbers more efficiently and tune their properties ex vivo [6,7]. In this chapter, we focus on recent advances in biomaterials-based tissue engineering studies of HSCs, such as hematopoietic niche mimic, functional regulation, and other potential applications. Moreover, we proposed the future scientific issues to be solved in advancing the preclinical translations.

Hematopoietic stem cells and hematopoietic stem cells niche

As the best characterized adult stem cells that had been identified nearly 60 years ago [8], HSCs own remarkable characteristics, including continuous self-renewal and differentiation into cells of all mature blood lineages [9]. Over past years, multiple surface markers have been used to identify, isolate, and purify HSCs [10]. For example, long-term HSCs (LT-HSCs) have been phenotypically defined as Lin-Sca-1 + c-Kit + Flt3 − CD34 − CD150 + CD48 − in mouse [11] and Lin-CD34 + CD38 − CD45RA − CD90 + CD49f + in human [12].

Due to progress in the understanding of the molecular mechanisms involved in regulating maintenance and expansion of hematopoietic stem/progenitor cells (HSPCs), a number of cytokines have been reported to play crucial roles in the proliferation, differentiation, and self-renewal of HSCs. These include stem cells factor (SCF), thrombopoietin (TPO), Angipoietin-1, Jagged-1, CXCL12 (also known as stromal cell-derived factor-1, SDF-1), IL-1, IL-3, IL-6, IL-11, IL-12, granulocyte colony-stimulating factor (G-CSF), and granulocyte−macrophage colony-stimulating factor (GM-CSF), which are reviewed in Refs. [13,14]. In addition to the hematopoietic cytokines, signaling pathways have also been shown in various models involved in the proliferation, differentiation, and self-renewal of HSCs, including Notch [15], Wnt [16], BMP [17], Hedgehog [18], Jak-Stats [19], Polycomb-group (PcG) proteins [20], and Homeobox (Hox) family [21].

HSCs are rare cells (about 0.005%−0.01%) normally located in BM [22], and they can migrate through the blood circulation into other tissues under certain conditions [23]. Two hematopoietic growth factors, G-CSF and GM-CSF, are commonly used as HSC mobilizing agents [24]. G-CSF treatment for mobilization of HSCs is the most efficient way to collect HSCs for clinical BM transplantation. Previous study demonstrated that murine HSCs increased within the central BM region after 4 days G-CSF administration, but not within the endosteal region [25]. This study suggested that HSCs respond differently to G-CSF, which is dependent on their locations. Homing of HSC to BM is also facilitated by chemokines [26]. The perivascular stromal cells of sinusoidal vessels produce CXCL-12 (SDF-1), while CXCR4 (receptor of CXCL-12) is expressed on HSCs. The SDF-1/CXCR4 pathway is essential for HSCs migrating from fetal liver to BM during development and plays a major role in directing the migration of

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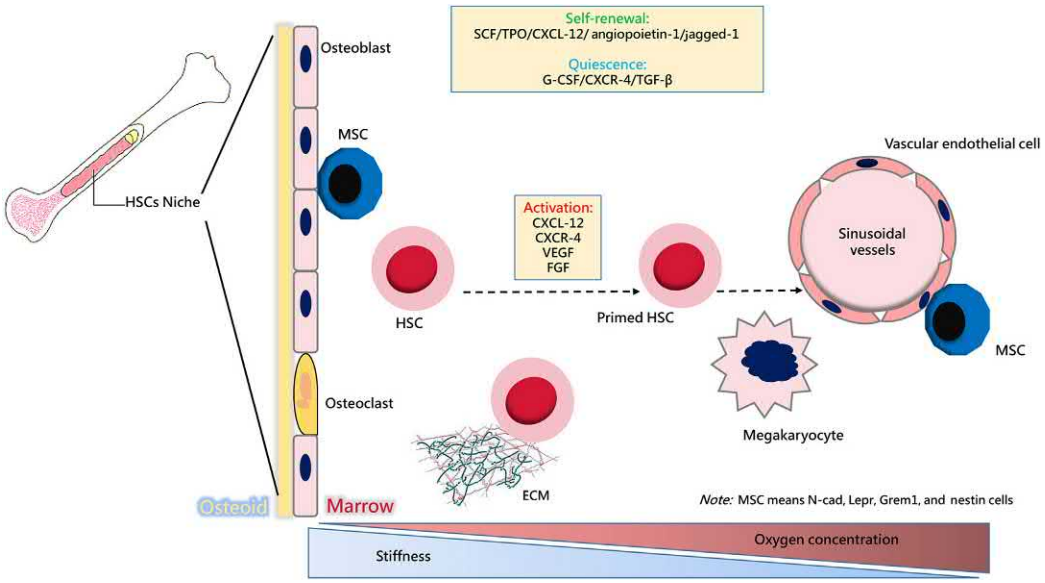

FIGURE 41.1 Schematic of the BM niche of HSCs. *BM*, Bone marrow; *HSCs*, hematopoietic stem cells.

HSCs [27]. Mobilization and homing processes provide theoretical basis for transplantation of HSCs.

HSCs transplantation have already been used in the clinic to treat various hematopoietic malignancies [28], such as leukemia [29], multiple myeloma [30], lymphoma [31], anemia, and myelodysplastic syndrome [32]. The source of transplanted HSCs can be isolated from BM, UCB, or mobilized peripheral blood of either patient's own (autologous) or a HLA-matched donor (allograft) [28], and the number of HSCs correlated with successful engraftment and patient survival [30]. High numbers of CD34 + cells (3×106/kg body weight) are clinically required for a successful transplantation, but the number of HSCs in a given graft may not be sufficient for transplantation proceeding [33]. Therefore methods for HSCs expansion ex vivo would be highly desirable.

However, HSCs rapidly differentiate and lose their self-renewal ability in vitro due to the lack of suitable microenvironment [34]. The local environment of HSCs in BM is referred to as stem cell niche, which is composed of extracellular matrix (ECM), stromal cells, and other cells (Fig. 41.1) [35,36]. Signals from niche components are essential for the regulation of HSC self-renewal and differentiation [37]. Cytokines including SCF, TPO, Jagged-1, SDF-1α, fms-related tyrosine kinase 3-ligand (Flt3L) are known molecular signals for self-renewal, quiescence, and engraftment in vivo [38–40]. These factors are usually secreted by other niche cells, such as osteoblasts, megakaryocytes, and perivascular cells (Table 41.1). ECM components distributed throughout the HSCs niche, such as fibronectin, collagens, and proteoglycans, can be sensed by HSCs through interaction with integrins, contributing to regulate HSCs [41–43]. Furthermore, ECM actively allows binding of cytokines and therefore favoring localization of HSCs [44,45]. Both cellular and extracellular components are key parts in the HSCs niche, and development of a functional hematopoietic microenvironment in vitro cannot ignore either of them.

Effects of biomaterials on hematopoietic stem cells

Biomaterials are natural or synthetic materials to replace or interact with biological system [60,61]. Extensive attempts to recreate HSCs niche by biomaterials in vitro had been reported (Table 41.2). As the HSCs niche is a fully 3D tissue, recent focus mainly aimed at developing 3D biomaterials to achieve niche-inspired signals. The use of biodegradable hydrogels for HSCs niche mimicking has increased, because they provide a 3D system that supports cell adhesion and mechanosensing [62,63]. Hydrogels can be made from natural (collagen [64], chitosan [65], hyaluronic acid [66], etc.) and synthetic (Polyethylene glycol (PEG) [67], Poly-L-lactic acid (PLLA) [68], etc.) polymers. Specific growth factors or molecules can be conjugated to hydrogel [69]. Conjugation of the PEG hydrogel with SCF and IFN-γ increased the total HSC population by maintaining stemness [70]. Another 3D platform for HSCs expansion is sponge-like porous scaffolds with interconnected network of pores to allow the exchange between nutrients and cells, and several materials have been used to construct porous scaffolds, such as collagen [71], PEG [72], and Polyurethane [73]. Moreover, nanofiber-based platforms have also been used to study HSCs. Carboxylic acid

TABLE 41.1 Cytokines in bone marrow niche affecting hematopoietic stem cells (HSCs).

Cytokines	Secreted niche cells	Effect to HSCs	References
SCF	Osteoblasts, MSCs	Enhances self-renewal and maintenance	[46,47]
TPO	Osteoblasts	Enhances self-renewal and maintenance	[21,48]
CXCL-4	Megakaryocytes	Inhibits self-renewal and induces quiescence	[49]
CXCL-12	Osteoblasts, CAR cells, perivascular cells	Enhances self-renewal	[50–52]
Angipoietin-1	Osteoblasts, MSCs, endothelial cells	Enhances self-renewal	[53,54]
TGF-β	Megakaryocytes, nestin + Schwann cells	Maintains quiescence and inhibits cell cycle	[55,56]
Jagged-1	Osteoblasts, perivascular cells	Enhances self-renewal	[39,46,57]
G-CSF	Endothelial cells, neutrophils	Induces quiescence and activation	[58,59]

SCF, Stem cells factor; *TPO*, thrombopoietin.

TABLE 41.2 3D platforms for hematopoietic stem cells (HSCs) expansion.

Polymers	Conjugated molecules or cells	Effect to HSCs	References
Collagen	BM niche cells	Expansion and myeloid differentiation depending on the hydrogel diffusivity	[75]
PCL	Collagen, fibronectin, fibrin	Promotes expansion, migratory, and adhesive property	[76,77]
PEG	RGD, SDF, Jagged-1	Promotes self-renewal and maintenance	[78,79]
PLLA	BM niche cells	Promotes long-term self-renewal	[80]
Decellularized bone	TPO, EPO	Promotes maintenance and expansion	[81]
Gelatin-methacrylamide	SCF	Increased LSK and differentiation potential	[82]
PVF	Collagen	Enhances expansion	[83]
PET	TPO, Flt-3L	Enhances expansion	[84]

BM, bone marrow; *PCL*, polycaprolactone; *RGD*, Arg-Gly-Asp; *PET*, positron emission tomography; *PVF*, polyvinyl formal; *SCF*, stem cells factor; *SDF*, stromal cell-derived factor; *TPO*, thrombopoietin; *EPO*, erythropoietin.

functionalized carbon nanotubes (CNT-COOH) was reported to increase the viability and support ex vivo expansion of HSCs in human UCB-derived mononucleated cells without adding any cytokine, particularly of CD45 + CD34 + CD38 − population and GM unit [74].

However, the safety and effectiveness of biomimicking materials to HSCs were often neglected. For maintaining clinical HSCs standard, both the starting and constructed materials should not show any negative effect on self-renewal, differentiation, migration, or cytotoxicity to HSCs [85]. But such data were wholly or partially absent in most of the related studies. In vitro expansion and differentiation potentials are major focused effects of biomimicking materials to HSCs. More completed data about interaction between HSCs and biomaterials-based ex vivo expansion system would promote us to design more suitable and effective expansion system for clinical application.

Applications

Engineering hematopoietic stem cells niche for in vitro expansion

Except for combining 3D platforms and cytokines (SCF, TPO, Flt3L, IL-3, etc.), other factors such as interacting cells and physical cues in engineering HSC niche have also been reported in the ex vivo expansion of HSCs. The coculture of HSCs with other niche cell populations, such as MSCs, osteoblasts, and megakaryocytes, is commonly

used to support HSCs growth and differentiation in vitro. In a previous study, HSCs were cocultured with mesenchymal stem cells (MSCs) and/or osteoblasts in decellularized bone scaffolds. The efficiency of expansion of HSCs in mixed cells (MSCs and osteoblasts) was much higher than those of individual MSCs or osteoblasts [86]. However, it is more difficult to separate HSCs from other cells in the mixed coculture system. Thus a noncontact coculture system should be established. For example, two types of cell can be placed on two sides with an efficient exchange of growth signals by using nanofiber platforms [87].

Moreover, modulation of physical factors, such as stiffness, dimensionality, and topography, has also been evaluated. Glycosaminoglycan was employed to modulate stiffness of hydrogel, and results showed HSCs formed less proliferative clusters in stiff cultures, compared to the soft hydrogel [88]. A range of fabrication cues can be used to manipulate architecture of scaffolds, such as electrospinning [89], lyophilization [90], and laser sintering [91]. Commonly, 3D culture systems have resulted in increase in HSC expansion compared to 2D cultures, and this was further enhanced by TPO and Flt3 supplementation [84]. In addition, fibronectin-immobilized positron emission tomography (PET) scaffolds led to a 100-fold expansion of human HSCs [92].

The most recent system for ex vivo expansion of HSCs is the BM-on-a-chip [93]. A poly(dimethylsiloxane) device with a central cylindrical cavity was microfabricated and filled with collagen gel and then perfused with culture medium in a microfluidic device. The engineered BM facilitates maintenance of a significantly higher proportion of LT-HSCs in vitro and retains hematopoietic stem and progenitor cells in normal in vivo-like proportions for at least 1 week. This technique provided an improved platform for producing HSCs naturally.

Considering the vasculature is critical in HSCs maintenance [94], the major caveat in mimicking HSCs niche is the lack of precise vascular structure. To overcome this issue the precise roles of each type of vessels should be explored. Moreover, the achievement of sustainable culture by developing vascular-on-a-chip should provide a clue [95] (Fig. 41.2).

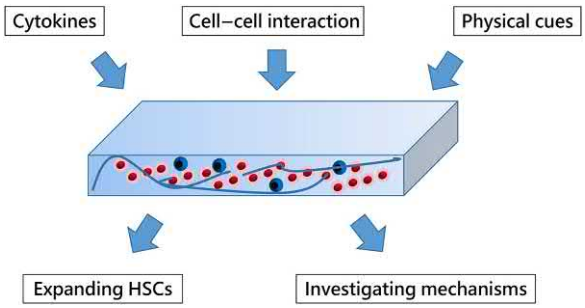

FIGURE 41.2 Current approaches for engineered HSCs niche. *HSCs*, Hematopoietic stem cells.

Manipulation of the multilineage differentiation of hematopoietic stem cells

By the construction of biomimic HSCs niche, we can also study the interactions between HSCs and the niche, more than just expanding clinical cell populations. BM is composed of two distinct niche (endosteal and vascular niches) that vary in a few properties including stiffness, types of feeder cells, oxygen concentration and distribution of cytokines [96,97]. The endosteal niche, closer to the trabecular bone, is home to quiescent HSCs by maintaining stemness, whereas primed HSCs are located more actively in the vascular niches [36]. Thus the differentiation of HSCs could be manipulated in vitro based on this theory.

In a previous study, when HSCs were seeded on a collagen hydrogel embedded with MSCs, HSCs' population migrated inside the hydrogel and presented a high level of primitive HSCs, while the HSCs that stayed above the hydrogel were differentiated [71]. Migrated HSCs received the majority of the cytokines provided by MSCs, while HSCs on the surface received much less, which suggested that the gradients of cytokines or other signals affect the fate determination of HSCs. Another study also reported that hydrogels with a high diffusivity can increase the paracrine signals between cocultured HSCs and Lin + fraction of BM cells, which caused the differentiation of HSCs into myeloid and lymphoid progenitor cells [75]. On the contrary, hydrogels with low diffusivity increased the number of primitive HSCs. Generally, a lower diffusivity required a higher concentration of starting materials, and thus a stiffer hydrogel promotes the stemness of HSCs because of the increased autocrine signals. Furthermore, 3D polyvinyl formal resin scaffolds with low oxygen led to maintenance of primitive HSCs, whereas hypoxia environment led to higher expansion [92]. In addition, feeder cells are commonly used to manipulate the differentiation into multiple lineage, such as myeloid, lymphoid, and megakaryocytic cells.

However, an adequate BM mimicking platform with the same variations between endosteal and vascular is challenging. Microfluidic-based systems have been widely used to generate gradient distribution of cytokines and ECM proteins in hydrogels [98]. A previous study demonstrated the effect of the gradient conjugation of SCF in hydrogel to the HSCs via a microfluidic system, and the gradient of cocultured osteoblasts and HSCs can also be generated [82,99]. Scaffolds with gradient in other cytokines and feeder cells should also be constructed and evaluated in future.

In vivo tracking hematopoietic stem cells

Although HSCs transplantation was successfully used in clinical therapies, numerous important issues remain lack of study. How many transplanted HSCs home into BM and contribute to hematopoiesis in a given time? How often the HSCs divide and how about their fate determination in vivo? Several groups have established different techniques to track the transplanted HSCs.

Many studies analyzed HSCs cell cycle by labeling cells with DNA dyes [100,101]. For example, BrdU was given orally or by intraperitoneal injection, and the kinetic of BrdU could be detected by fluorescent-activated cell sorting to provide information about cell division [102]. By doing this, it is estimated that about 8% of a population enriched by surface marker of HSCs randomly enter into cell cycle per day, and all HSCs enter into cell cycle on every 57 days. However, BrdU-mediated DNA labeling requires cell fixation, which prohibits testing of the functionality of labeled HSCs. Methods for labeling cell surface or cytoplasmic protein can trace cells real time; however, labeled cells would be diluted as cell division [103,104]. For example, a previous study reported biotin−avidin-labeled HSCs were mainly found in BM after 1 week, and primitive HSCs showed higher label signals than other mature hematopoietic cells [105]. However, low divisional resolution could distinguish maximally to only four divisions, which limited the long-term in vivo tracking.

In addition, current approaches for HSCs in vivo tracking were based on bioluminescence imaging (BLI) [106]. However, the limitation of light transmission through tissue restricts the applications of BLI in small animal models, because light signal can hardly penetrate large tissues in human [107]. In contrast, magnetic resonance imaging (MRI) can provide high penetration signal and 3D information, which appears to be a useful way for in vivo tracking of HSCs [108]. Magnetic labeling is required in MRI, and the magnetic contrast agents are safe to HSCs. The same goes for nuclear imaging (NI), such as PET and single-photon emission computed tomography, but the use of radioactive substances which lead to genotoxicity lacks study. However, multimodality imaging combines BLI, MRI, and/or NI, which provides dual benefits of high sensitivity and resolution [109].

Future perspectives

Over past decades, growing evidences indicated that HSC expansion in vitro might actually be achievable. This is based on a better understanding of HSCs niche and niche-HSCs interaction for regulating HSCs in vivo, which have led to novel strategies to expand HSCs ex vivo. However, we are currently in an early stage for moving forward the clinical applications of HSCs-based tissue engineering, and there is still abundant future work to be completed.

First, the safety and effectiveness of biomimicking materials to HSCs should be focused to meet clinical standard. Next, combination of 3D scaffolds, cytokines, feeder cells, and appropriate physical condition might provide a robust option for HSCs expansion ex vivo. Engineered HSCs niche also offered a tool to study the interaction mechanisms of HSCs and niche in vivo. Extensive mechanistic studies would further guide the design of engineered HSCs niche, which is necessary as a first essential step for clinical translation. Besides, more clinical approved contrast agents can be used in HSCs labeling for in vivo tracking real time, and multimodality imaging methods should be developed. Despite the numerous challenges faced when exploring the clinical translations, more in vitro and in vivo experiments are still essential to reduce clinical cost, and all these efforts will accelerate the development of HSCs-based tissue engineering in the future.

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Chapter 42

Blood components from pluripotent stem cells

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Introduction and history of modern hematology

Hematopoiesis is a highly regulated, dynamic process for the generation and maintenance of all mature blood cell types. The discovery of the major cell types within the blood and their importance for human health was recognized many centuries ago. In the mid-1600s, Jan Swammerdam first reported the presence of red blood cells (RBCs) while examining samples under the microscope. Two hundred years later, in the mid-1800s, French physician Gabriel Andral and British physician William Addison first reported the discovery of white blood cells (WBCs). During this same era, French physician, Alfred Donne described the discovery of platelets [1]. Initial attempts to transfuse blood between humans occurred in the early 1800s; James Blundell first experimented with transfusing blood in up to 10 patients [2]. Even though little was known about blood typing at the time, he was successful in half of these cases, which prompted others to continue exploring the practice. In the early 1900s the Austrian physician, Karl Landsteiner, discovered the A, B, and O blood groups and the importance of matching blood types for successful and safe transfusion. He also helped discover the Rhesus (Rh) blood group system as another important consideration for ensuring safe transfusions [3]. These discoveries helped lead to further improvements in blood typing, collection, storage, fractionation, and transfusion practices over the course of the 20th century to treat various blood disorders, including hemophilia, hemorrhaging bleeding, and anemia. In the 1950s, both chemotherapy and bone marrow (BM) transplantation emerged as therapeutic options to treat hematologic malignancies. In 1956, Thomas was the first to successfully perform BM transplantation; he treated a boy with leukemia using BM collected from the boy's identical twin [4]. Later research highlighted the importance of and guidance for human leukocyte antigen (HLA) matching between donor and recipient for success of BM transplant and avoiding immune rejection and graft versus host disease [5]. It is now recognized that the hematopoietic stem cell (HSC) is the most critical component of BM for transplantation as it can give rise to all mature blood cell types. Today, more than 50,000 HSC transplantations (HSCTs) are performed each year [6], highlighting the importance of this procedure for treating hematologic diseases.

Long-term HSCs reside in the BM and sit at the top of the hematopoietic hierarchy. They give rise to short-term HSCs, which then further differentiate into early stage myeloid and lymphoid progenitors (Fig. 42.1).

Extrinsic cytokines and growth factors stimulate transcription factor activity to further drive lineage-specific differentiation programs, and through a multistep process, eventually generate the diversity of cell types seen in the peripheral blood (PB). Myeloid lineage progenitors give rise to erythrocytes, megakaryocytes (MKs)/platelets, monocytes, and granulocytes, while lymphoid lineage progenitors give rise to T, B, NK, and NKT cells (Fig. 42.1). As we discuss below, these cells each play a unique and vital role in maintaining human health; therefore the ability to replace or repair elements of the hematopoietic system during injury or disease is a major medical focus.

Red blood cells

Erythrocytes or RBCs are the most abundant cell type in the PB with an average of 5×10^{12} cells/L (Fig. 42.2). They represent 40%–45% of the total blood volume and have the critical role of binding and transporting oxygen

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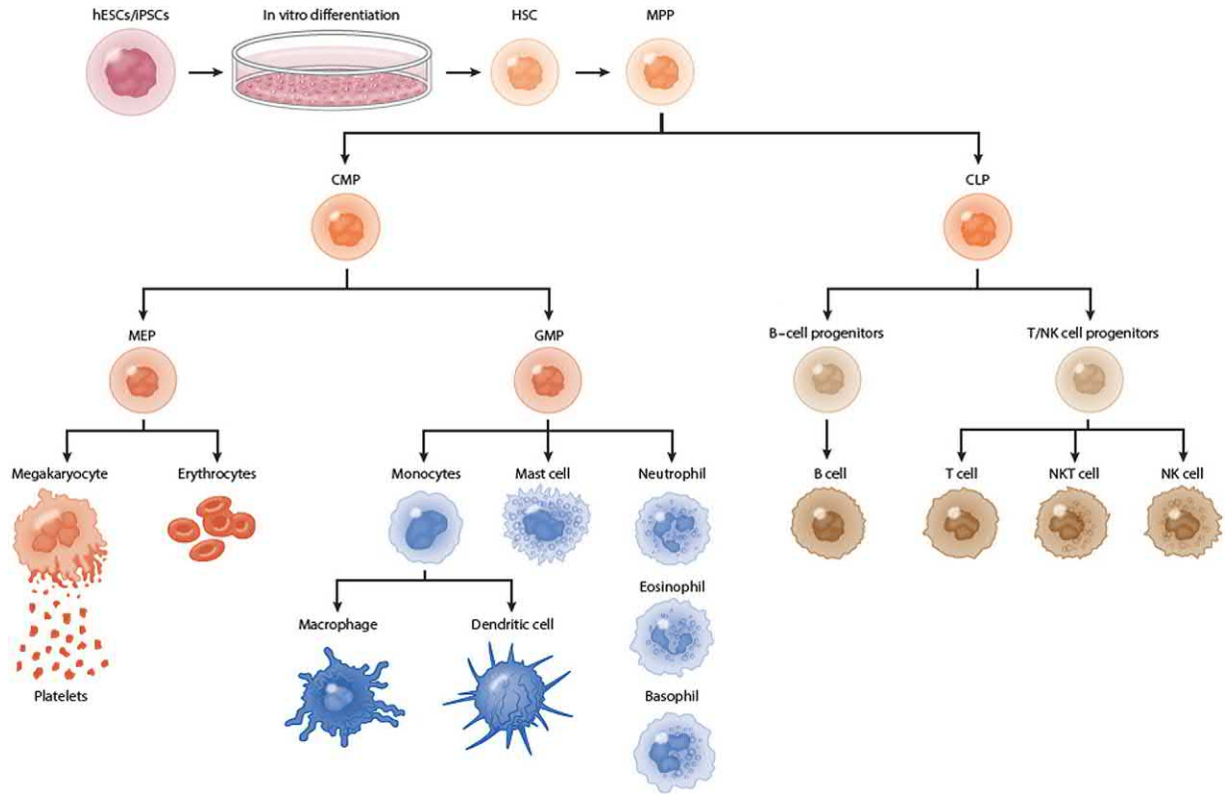

FIGURE 42.1 Hematopoietic hierarchy. All mature blood cell types within the adult hematopoietic system arise from HSCs in the bone marrow. hESCs/iPSCs can be induced to differentiate down the hematopoietic lineage in vitro with the goal of producing blood components that mimic those found naturally in the body. *hESCs*, Human embryonic stem cells; *HSC*, hematopoietic stem cell; *iPSCs*, induced pluripotent stem cells.

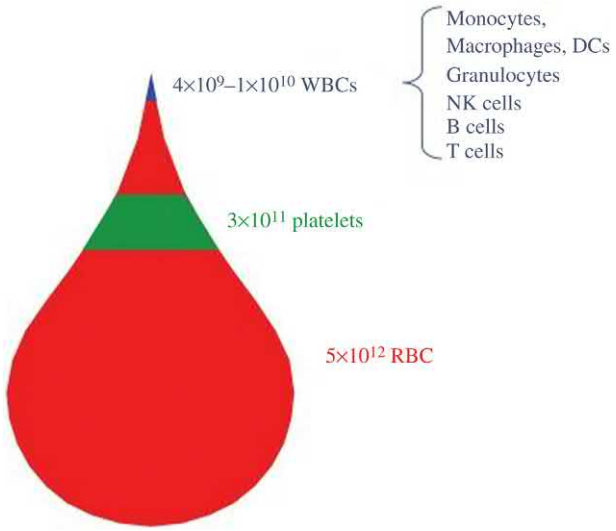

FIGURE 42.2 Number and types of cells per liter of human peripheral blood. PSCs can be differentiated toward these mature blood cell types in vitro. Ongoing efforts are aimed at improving the efficiency of differentiation and/or the maturation of PSC-derived blood components. *PSCs*, Pluripotent stem cells. *Reprinted with permission from Kimbrel EA, Lu SJ. Potential clinical applications for human pluripotent stem cell derived blood components. Stem Cells Int 2011;2011:273076.*

to tissues of the body. More than 14 million units of whole blood and apherised RBCs were collected for transfusion purposes in the United States in 2013 according to the National Blood Collection and Utilization Survey. This represents a 9% decline from previously reported numbers in 2011, likely due to improved patient blood management practices and alternative medical options for patients that used to be treated with transfusions [7]. Despite this trend, there is still a great demand for blood donation for the purpose of replacing RBCs in patients that have lost significant amounts of blood or are severely anemic. Moreover, universally immunocompatible blood, that is, (O) Rh − is perennially in short supply due to the low frequency of this blood type in the general population, with less than 8% of people in Western countries and less than 0.3% of people in Asia with this type.

RBCs contain hemoglobin, a 4-subunit, 4 heme-group containing protein that binds iron, which in turn binds oxygen. During early development, primitive, yolk-sac-derived RBCs contain embryonic hemoglobin (hemoglobin ε, epsilon). Further embryonic development leads to the initiation of definitive hematopoiesis within the fetal liver and the production of enucleated RBCs containing

fetal hemoglobin (HbF). The transition from fetal to adult globin occurs within the first few months of life [8]. HbF comprises two alpha and two gamma subunits, while adult hemoglobin (HbA) contains two alpha and two beta globin subunits. Sickle cell anemia and beta-thalassemia are two commonly inherited blood disorders that involve defects in HbA. Beta-thalassemia involves a defect in the production of beta globin, leaving its alpha counterpart unpaired, causing the death of erythrocyte precursors. Sickle cell disease involves the mutation of the beta globin gene, which causes hemoglobin to polymerize and distort RBCs into a "sickle" or crescent shape. These abnormally shaped cells can lead to clogging in blood vessels, pain, and impaired oxygen delivery to tissues. It is estimated that 300,000 children are born with these diseases each year, translating to several million people living with these disease worldwide [9]. In both situations, transfusion of normal RBCs can help.

In the adult BM, erythropoiesis involves the differentiation of the common myeloid progenitor (CMP) to an MK−erythroid progenitor, which then further differentiates and gives rise to the lineage-restricted preerythrocyte, followed by early, intermediate, and late erythroblast stages. At this point, the nucleus is extruded, and the cell is called a reticulocyte. Reticulocytes enter the PB, further mature into an RBC, and circulate for ~120 days prior to being phagocytosed by macrophages [10]. Stem cells within cord blood (CB), PB, and BM can all be induced to generate erythrocytes through cytokine-driven in vitro culture. Supplementation of culture media with erythropoietin (EPO), stem cell factor (SCF), and interleukin-3 (IL-3) facilitates this differentiation; coculture with stromal feeders, such as MS-5 or human mesenchymal stromal cells (MSCs), helps facilitate enucleation [11−13]. In a recent example, CB-derived CD34 + cells were differentiated ex vivo into RBCs using a four-step differentiation protocol. Here, SCF, thrombopoietin (TPO), and fms-like tyrosine kinase 3 (Flt3) ligand helped with initial expansion, while the combination of SCF, EPO, IL-3, granulocyte−macrophage colony stimulating factor, and Flt-3 ligand (FL) was used in the last stage of the 21-day protocol to facilitate the expansion and maturation of erythrocyte progenitors. Using this method, 1 million CD34 + CB cells gave rise to 2.9×10^{11} RBCs, with 90% expressing CD235a and 50% enucleated. Upon injection into immunodeficient NOD/SCID mice treated with sublethal irradiation, these RBCs survived for 3 days, similar to primary human RBCs, further matured, and enucleated. In a nonhuman primate model of hemorrhagic anemia, these RBCs enhanced hematologic recovery without adverse effects [14]. Only one study has tested clinical use of ex vivo produced RBCs. Here, PB CD34 + cells were differentiated into RBCs and reinfused into the patient without adverse effects [15]. These approaches, while feasible, are still limited by their donor-dependent sources and are expensive to produce for autologous use.

Pluripotent stem cells (PSCs) may serve as an off-the-shelf alternative to donated or ex vivo derived RBCs, particularly if starting from a universally immunocompatible (O) Rh − PSC line. Early studies have demonstrated the feasibility of using human embryonic stem cells (hESCs) for this purpose [16−20], and additional studies have shown similar proof of concept using induced PSCs (iPSCs) [21−24]. For example, in one such study, our group demonstrated that it was possible to obtain functional, oxygen-carrying erythrocytes from various starting PSC lines on a large scale [17] (Fig. 42.3). Another approach involves the use of ectopically expressed transcription factors to drive direct conversion of fibroblasts to hematopoietic progenitors and further differentiation into erythroid cells [25,26] or conversion of B cells to erythroid lineage cells [27]. Although several different approaches were used in the above studies, none were able to completely overcome two issues: inability of most cells to express beta globin (i.e., inability to undergo "globin-switching") and inefficient enucleation.

More recent studies are beginning to unravel the intricacies of these cellular events and finding ways to overcome the hurdles. Proteomic comparison of iPSC−RBCs with adult RBCs showed that although there was considerable overlap in the expression of more than 30 key erythroid markers, there were differences in the expression of cytoskeletal proteins, which the authors suggest could account for inefficient enucleation of the iPSC-derived RBCs [28]. In other studies, gene expression signatures during iPSC differentiation of RBCs were compared to that of adult, CB progenitors and/or K562 erythroleukemia cells. The pattern of gene expression for iPSCs differentiating into RBCs differed from these comparators. Among the differences, the key erythroid markers, Sox6, MYB, BCL11A, and KLF1 were downregulated, while embryonic hemoglobin ε was upregulated in iPSC derived versus the other sources of erythroid cells [29,30].

A strategy to overcome the block in globin switching for PSC-derived RBCs may be manipulating the expression levels of factors involved in globin switching [31,32]. In one study, increasing expression of BCL11A in an iPSC erythroid cell line called HiDep-1 was helpful for increasing beta globin levels, as BCL11A binds to locus control region and intergenic regions between gamma and beta globin to upregulate beta globin. This strategy worked in HiDep-1 cells likely because they already express high levels of KLF1, another critical factor involved in globin switching [29]. In another study, manipulation of globin switching regulatory genes promoted reversion of beta globin to gamma. Here,

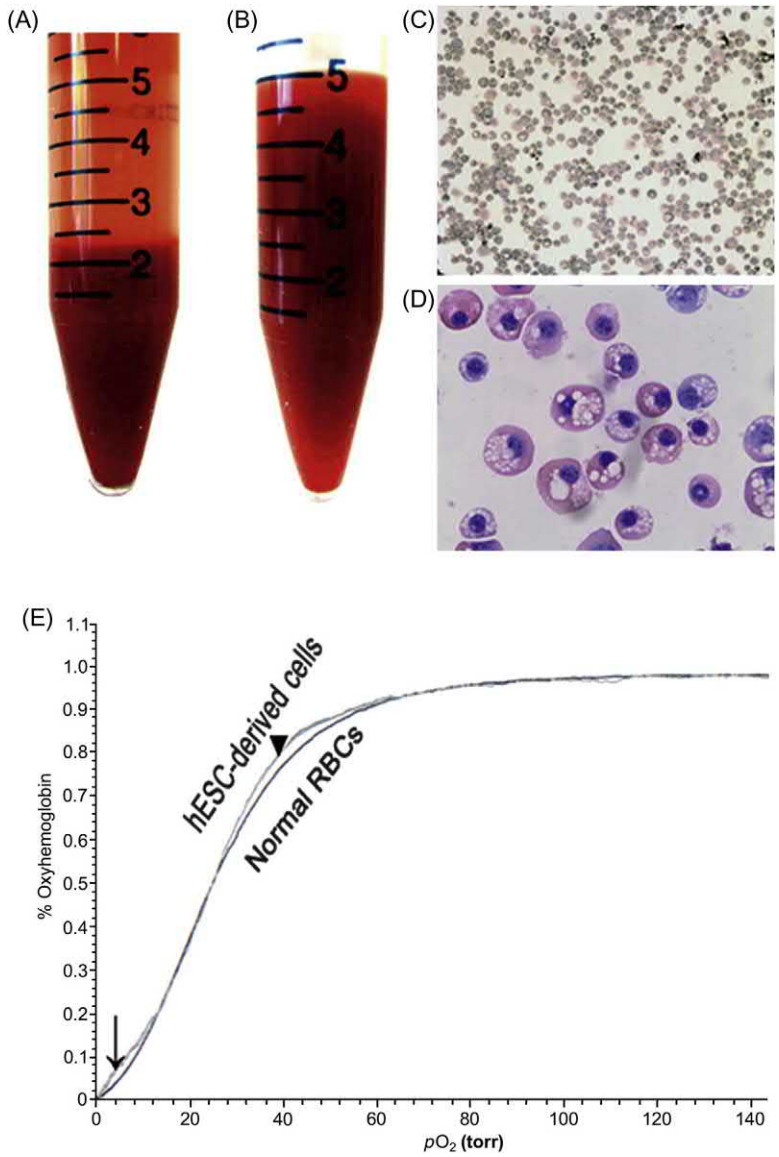

FIGURE 42.3 Large-scale production of erythroid cells from hESCs. (A) Erythroid cells (pellet) derived from 2×10^6 hESCs; (B) erythroid cells from panel a were resuspended in equivalent hematocrit of human whole blood; (C and D) morphology of erythroid cells derived from hESCs (C, ×200; D, ×1000); (E) functional characterization of hESC-derived erythroid cells. Oxygen equilibrium curves of normal human erythrocytes and hESC-derived erythroid cells. The two curves are virtually indistinguishable at their midpoints, whereas the curve of hESC-derived erythroid cells are leftward shifted at low (→) and high (▼) oxygen saturation percentages. *hESCs*, Human embryonic stem cells. *This research was originally published in Lu SJ, et al. Biologic properties and enucleation of red blood cells from human embryonic stem cells. Blood 2008;112:4475–84. © The American Society of Hematology.*

knockdown of mi2beta increased gamma globin expression in erythrocytes derived from CD34+ progenitors [33]. Mi2beta is a component of the NuRD complex normally involved in enhancing expression of KLF1 and BCL11A. Reducing levels of mi2beta or BCL11A decreases beta globin while increasing gamma globin, which is a useful therapeutic strategy for sickle cell disease or beta thalassemia [34]. Further work to manipulate this system in the opposite direction may be beneficial for driving beta globin expression and reducing gamma globin in PSC–RBCs. Toward this end, Yang et al. used a tamoxifen-ER-inducible system to drive EKLF/KLF1 at the AAVS1 safe-harbor locus in iPSC differentiating to erythrocytes. This slightly enhanced enucleation, shape, and stability of the resulting RBCs by increasing the generation of CD71+/CD235a+ cells from 30% up to 65%. Yet surprisingly, it did not induce globin switching to beta globin [35], suggesting that further work still needs to be done to optimize globin switching. In another study, an ES-sac culture method was used to enhance RBCs from hESC/iPSC, increasing the expression of beta globin over time, going from 0% at day 9 to 18% at day 18 [36]. In another study the use of microcarriers followed by coculture with MSCs helped drive a 6-fold increase in hematopoietic progenitors and an 80-fold increase in RBCs with improved maturation and enucleation [37]. Continuing efforts like these are still focused on improving beta-globin switching, enucleation, and importantly, large-scale manufacturing to enable clinical application of PSC–RBCs.

Megakaryocytes/platelets

MK are large (~50−150 μm in diameter), polyploid (up to 128N) cells that generate platelets. Platelets are the second most abundant cell type in the blood, with $\sim 3 \times 10^{11}$/L (Fig. 42.2). These small, disk-shaped, anucleate cell fragments play a key role in thrombogenesis (i.e., clot formation), hemostasis, and vascular repair [38]. Compared to RBCs, platelets are relatively short lived, lasting only 7−9 days in circulation. Although each MK has the capacity to generate between 2000 and 10,000 platelets, thrombocytopenia, a condition where counts drop below 150,000/μL (1.5×10^{11}/L) may occur if platelet production is impaired [39]. This is often the case in patients with liver failure or leukemia. Thrombocytopenia may also occur if platelets are destroyed, as is the case during chemotherapy. Low platelet counts can be quite serious as it may put a person at risk of uncontrolled bleeding with a fall or skin laceration. Transfusion of platelets can help alleviate thrombocytopenia but high demand and limited shelf life put transfusable platelets in constant demand. Moreover, refractoriness, largely due to HLA alloimmunization occurs in ~1/3 of transfusion patients [40]. The use of PSCs as an alternative source of platelets to donated blood may help alleviate these issues.

Proof of principle for the use of hESCs dates back to 2008, where an ESC-derived sac-like structure gave rise to hematopoietic progenitors, which further differentiated into MK and platelets through the use of TPO, SCF, and heparin [41]. In another method developed by our group, hemangioblasts derived from hESCs were used in a serum and feeder-free method to drive MK and platelet production [42]. Here, differential interference contrast and electron microscopy showed that ultrastructural and subcellular features of hESC-platelets were identical to those of primary, adult PB platelets. Various in vitro assays demonstrated that these hESC-platelets have functional properties similar to those of PB platelets as well, including responsiveness to thrombin stimulation, microaggregation, and clot formation/retraction upon stimulation with physiological activators (Fig. 42.4). Moreover, these hESC-platelets participated in developing mouse platelet thrombi at the site of laser-induced arteriolar wall injury in vitro similar to normal human PB platelets [42]. iPSCs have also been used to generate MKs and platelets with these two initial methods [43,44] to a similar degree as with hESCs. However, with less than 20 platelets

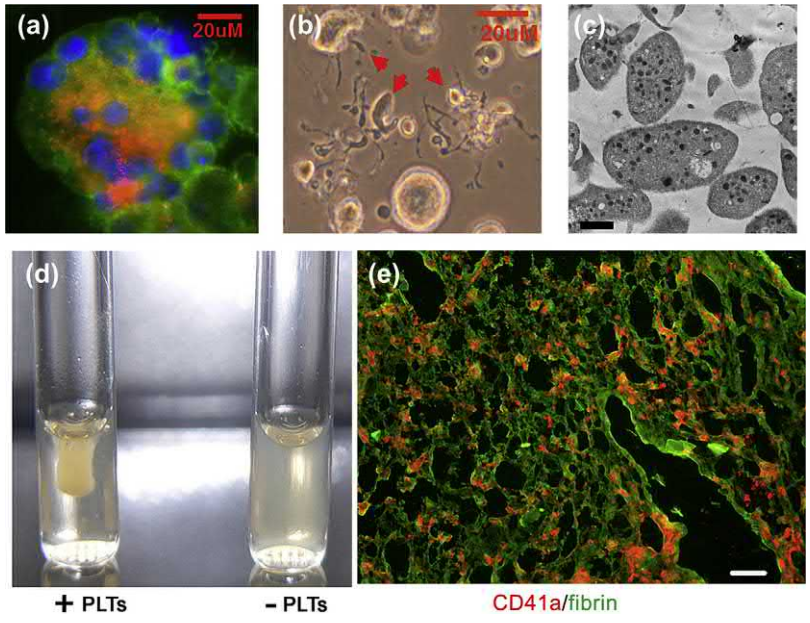

FIGURE 42.4 Generation and characterization of MKs and platelets derived from hESCs. (A) Immunofluorescence of vWF (red) and CD41 (green) proteins in MKs derived from hESCs. vWF is localized in the cytoplasm in a punctate pattern, which is typical for MKs. CD41 is expressed on the surface. 4′,6-diamidino-2-phenylindole (DAPI) (blue) stain shows polynuclei (polyploidy); (B) phase contrast image of proplatelet forming MKs derived from hESCs; (C) thin-section transmission electron microscopy of platelets generated from hESCs, Bar = 1 μm; (D) in vitro functional characterization of platelets generated from hESCs. Platelet-depleted human plasma was added with (+PLTs) or without (−PLTs) hESC-platelets (1.5×10^7/mL), and thrombin (2 U/mL) and CaCl2 (10 mM) were then added to the suspensions to induce clot formation/retraction. No clot formation/retraction was observed without addition of hESC-platelets (−PLTs); (E) clot cryo-sections were stained with antihuman CD41 (red) and antihuman fibrin (green) antibodies. Images were taken under a fluorescence microscope. Bar = 50 μm. *MKs*, Megakaryocytes; *hESCs*, human embryonic stem cells; *vWF*, von Willebrand factor. *This research was originally published in Lu SJ, et al. Platelets generated from human embryonic stem cells are functional* in vitro *and in the microcirculation of living mice. Cell Res 2011;21:530−45.* © *IBCB, SIBS, CAS.*

coming from each MK, the production is still several orders of magnitude less than the 2000–10,000 platelets per MK made in the body.

To enhance generation of platelets from iPSC-MKs, Thon et al. report the use of a shear force system to recapitulate their native BM environment [45]. This system nearly doubled the number of platelets per MK that had previously been reported, yet it still fell short of physiologically relevant numbers. In another study, Moreau et al. used three transcription factors (GATA1, FLI1, and TAL1) to drive megakaryocyte commitment, starting with a 10-day-cytokine–supplemented, embryoid body (EB)-based differentiation system. EBs were subsequently dissociated into single cells and plated onto tissue culture–treated plates. At this stage, supplementation with low TPO and SCF helped maintain cultures for 90+ days with enhanced MK yield and purity, generating up to 2×10^5 MK per input iPSC. This was a noted improvement over the 1300 MKs per iPSC yield that was achieved when using high TPO and IL1β supplementation [46]. Importantly, the optimized protocol enabled large scale expansion and cryopreservation of MKs. Platelets derived from such optimized MKs participated in clot formation in vivo in a laser-induced vascular injury model, yet similar to other studies, the yield of platelets was still much lower than that achieved in vivo. Direct conversion approaches have also been explored for generating MKs and platelets from primary cell sources without having to go through a pluripotent stage. Pulecio et al. describe the use of a six-transcription factor cocktail consisting of GATA1, GATA2, RUNX1, TAL-1, LMO2, and c-MYC to directly convert fibroblasts to CD41+ MK cells. These could further give rise to platelets in vitro or upon transplantation into mice [47]. Another group reported the use of p45NF-E2, MAF G, and MAF K to convert human dermal fibroblasts into CD41+ MKs. In vivo transplantation of these "iMKs" produced platelets, which upon recovery could participate in an ex vivo thrombus formation assay [48]. Improvement in MK production from CD34+ cells has also described using a three-step system involving increasing pH, oxygen content, and differential application of cytokines. High ploidy MKs were produced, yet platelet production was still inefficient, suggesting that the in vitro culture environment rather than issues with the starting material may be responsible for low platelet production [49]. Newer culture systems are needed to augment in vitro production of platelets in more physiologically relevant quantities from MKs. In addition, further work is needed to determine how best to isolate, increase shelf-life, and ship platelets on a commercial scale without damaging their functional properties.

White blood cells

WBCs represent only ~1% of circulating cells in the PB (Fig. 42.2), yet they play extremely important roles in providing both innate and adaptive immunity against viruses, bacteria, parasites, and the outgrowth of cancer. Three main classes of WBCs are lymphocytes [containing T, B, NK, NKT, and plasmacytoid dendritic cells (DCs)], monocytes (which can differentiate into macrophages and myeloid-derived DCs), and granulocytes (containing neutrophils, eosinophils, basophils, and mast cells). Lymphocytes arise from the progressive differentiation of common lymphoid progenitors (CLPs), while granulocytes and monocytes arise from the CMP, as mentioned previously (Fig. 42.1). Several studies have shown that hESCs and iPSCs can differentiate into both lymphoid and myeloid lineage WBCs, and current efforts are underway to develop these PSC-derivatives as off-the-shelf therapies to bolster innate and adaptive immune responses, as summarized later.

Lymphocytes—T cells

T cells are present at a concentration of 1×10^9/L of PB and represent about 10% of WBCs, or 0.1% of all circulating cells. As part of the adaptive immune system, T cells can be stimulated to mount antigen-specific immune responses against pathogens and cancer cells. While a detailed review of T cell biology is beyond the scope of this chapter, in brief, T-cell precursors arise from CLPs in the BM and migrate to the thymus for further differentiation and maturation. Mature T cells can be classified into six main types based on their unique immunophenotype and function: helper CD4+ T cells that secrete specific cytokines in response to MHC II-presented antigens; cytotoxic CD8+ T cells that release cytotoxic enzymes in response to MHC I-presented antigens; long-lived CD4+ or CD8+ memory T cells; immunosuppressive CD4+CD25+FoxP3+ regulatory T cells (Tregs); skin, gut, or lung-resident γδ T cells; and rare, CD1d-restricted NKT cells. Clinical use of primary T cells dates back to the late 1980s when it was found that autologous T cells could be isolated, expanded ex vivo, and then adoptively transferred back into patients to treat melanoma [50]. Since then, many improvements have been made and are still being made to increase the utility, safety, and efficacy of adoptive T cell therapy (ACT) protocols [51–54].

Arguably, the most significant improvement made to ACT has been the creation and use of engineered, chimeric antigen receptors (CARs) to increase specificity against tumor antigens. First, CAR technology enables antigen-specificity to be delivered in an MHC-independent manner and second, it obviates the need for

isolating patient-specific, tumor-infiltrating T-cell clones. The concept of using CARs to provide greater antigen specificity for target cell killing was first proposed in 1989 by Gross et al. Here, they fused the antibody variable heavy and light chains (Fab′ fragments) to the T-cell receptor (TCR) constant domain to create a chimeric TCR [55]. This chimeric TCR conferred antigen-specific IL-2 production and cytolytic responses from the engineered cells to cellular targets displaying 2,4,6-trinitrophyenyl haptens. Further refinement of this engineering approach in 1993 led to the use of a single-chain variable region, single-chain variable fragment (scFv) fused through a spacer and transmembrane domain to the gamma (γ) or zeta (ζ) chains of CD3 for downstream signaling [56]. This simplified design employing scFv and a CD3 ζ chains is viewed as the first generation of modern CAR technology. First generation, anti-CD20 CAR T cells were tested in clinical trials for non-Hodgkin lymphoma and mantle cell lymphoma [57] and first generation anti-alpha folate receptor CAR T cells were tested in clinical trials for ovarian cancer [58]. Neither of these trials showed much clinical success and thus first generation CARs were quickly replaced with second generation versions containing costimulatory domains such as CD28 [59,60] or 4-1BB [61,62]. Second generation CAR T cells have proven much more efficacious than their first generation counterparts with numerous clinical studies reporting efficacy using anti-CD19 CAR T cells to target B-cell malignancies. These include relapsed or refractory acute lymphoblastic leukemia (ALL) in both pediatric and adult cases, diffuse large B-cell lymphoma, chronic lymphocytic lymphoma, and follicular lymphoma [63–72]. Success with second generation CAR T cell therapy led to the approval of tisagenlecleucel (Kymriah, Novartis) in August 2017 for B-cell ALL in patients up to 25 years of age, followed shortly thereafter with the approval of axicabtagene ciloleucel (Yescarta, Kite Pharma) in October 2017 for adults with relapsed or refractory diffuse large B-cell lymphoma. Kymriah's approved indications were expanded to include adult relapsed or refractory diffuse large B-cell lymphoma in May 2018. The most common side effects reported for CAR T cell therapies include cytokine release syndrome (CRS) and neurologic toxicity. CRS involves increased release of proinflammatory cytokines by the activated CAR T and other immune cells within close proximity. It can be controlled with steroids and anti-IL-6 therapy, while corticosteroids are often given to deal with neurologic toxicity even though it is generally more difficult to control than CRS [73].

The CAR technology field is still rapidly evolving. Antigen specificity of CARs in clinical trials has now greatly expanded beyond CD19 to include other hematologic targets such as CD22 for leukemias and lymphomas and BCMA for multiple myeloma. In addition, CARs with specificity against solid tumor antigens are also in clinical trials. Examples include epidermal growth factor receptor (EGFR) for treatment of glioblastoma, mesothelin for pancreatic, cervical, breast, and hepatocellular cancers, and MUC1 for lung, gastric, pancreatic, and colorectal cancers (these and others reviewed in Ref. [74]). Moreover, further improvements to CAR design features have led to the creation and subsequent clinical testing of third generation CAR technology. This involves using two additional costimulatory domains, such as various combinations of CD28, 4-1BB, ICOS, OX40, and/or CD27 in addition to the standard CD3ζ in order to increase the persistence of CAR T cells within circulation [75–77] (Fig. 42.5).

Fourth generation CAR technology has also been in development and involves transgenic expression of proinflammatory cytokines such as IL-12 and IL-15 to augment antitumor efficacy of second generation CARs. Fourth generation CARs are also referred to as T cells redirected for universal cytokine killing, or "TRUCKs," and may be helpful for targeting heterogeneous tumors that are difficult to kill with a single targeted CAR moiety or as a therapeutic option to treat diseases other than cancer, such as autoimmune or metabolic disorders [78]. Next generation

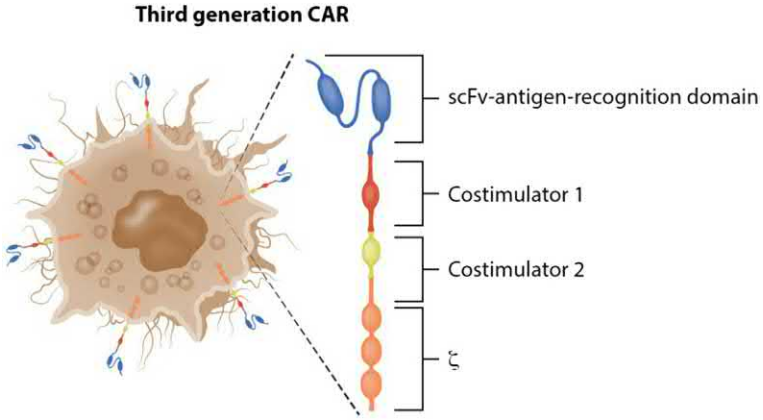

FIGURE 42.5 Features of third generation CAR constructs. An scFv antigen-recognition domain is coupled to two costimulatory domains, followed by the intracellular signaling domain of CD3, zeta (ζ) chain. CAR technology is now being applied to PSC-lymphocyte derivatives. *CAR*, Chimeric antigen receptor; *PSC*, pluripotent stem cell; *scFv*, single-chain variable fragment.

CAR technology goes beyond the fourth generation to improve the scope, precision, and utility of CAR technology through at least nine other types of engineering approaches. Examples include "self-driving" CARs that involve the use of a chemokine receptor to promote better tumor binding, "armored" CARs whose engineering makes them resistant to immunosuppression, and "self-destruct" CARs that use transient mRNA transfection of the CAR gene to limit the expression of the CAR instead of using a more conventional, integrating, retrovirus-based approach. Another approach to limit CAR expression involves the use of an inducible suicide gene, such as iCasp9 or CD20 to control persistence of the CAR-containing cells upon administration of a small molecule or antibody. Other engineering approaches include the "conditional" CAR, whose full activation is only achieved upon providing a small molecule as well as the "tanCAR" or tandem approach which involves the use of two scFvs with different affinities to provide more specific targeting of tumors and avoiding on-target, off-tumor effects (these and others reviewed in Ref. [79]). Nonengineering approaches are also being developed to increase CAR T-cell persistence. These include the use of lymphodepletion to remove Tregs, infusion of cytokines to support the viability of CAR T cells in vivo, and the use of T cells that are specific for chronic viral infections [e.g., cytomegalovirus (CMV) or Epstein–Barr virus (EBV)] as the starting material for CAR T-cell generation. In the last example, vaccination with CMV or EBV viral peptides has been found to stimulate the proliferation of the resulting CAR T cells for longer term specificity against a tumor antigen, such as CD19. These approaches are being tested in clinical trials as a way to enhance long-term survival of CAR T cells [80].

Most studies to date have relied on the use of autologous cells for CAR T cell therapies; however, the use of autologous cells is very expensive and also requires time for individual engineering of the CAR cells. Use of allogeneic cells would help with the production of off-the-shelf CAR-engineered cells, drive down costs, and make these therapies immediately available for patients in need. A couple of studies have already explored the use of allogeneic CAR T cells clinically and surprisingly, found they could be used without eliciting graft versus host disease [81,82]. This may be due to the rapid stimulation and immediate exhaustion kinetics of first generation CAR T cells [83]. These studies also pave the way for alternative allogeneic sources, such as PSCs, to be considered as starting material.

The first studies demonstrating that PSCs can differentiate into T cells were published in the mid-2000s. Galic et al. published two papers in 2006 and 2009 showing that hESCs, cultured either on OP9 stromal cells or as EBs with hematopoietic cytokine cocktails, could be induced to differentiate into CD34 + hematopoietic progenitors capable of giving rise to T cells upon their transplantation into a Thy/Liv organ implant within SCID or RAG2 deficient mice [84,85]. In the EB-based method, the Thy/Liv implant environment facilitated the generation of CD4/CD8 double positive T cells within 4 weeks and single positive CD4 and CD8 T cells that had undergone TCR rearrangement by 8 weeks after transplantation [85]. Subsequent studies by Timmermans et al. showed that hESCs could differentiate into mature T cells using a completely in vitro system. Here, hESCs were cultured on OP9 to produce hematopoietic progenitors and then transferred onto OP9 cells expressing delta ligand 1 (DL1) for another 5–7 weeks with FL, SCF, and IL-7 supplementation. Within 30 days, CD3 + T cells that had undergone TCR rearrangements emerged and could be induced to secrete IFNγ in response to PHA induction, similar to primary T cells [86]. Similarly, Kennedy et al. used an EB-based method to isolate $CD34^{+}$, $CD43^{lo/-}$ progenitors and could induce their differentiation toward T-cell lineage by coculturing them on OP9 expressing DL4. Here, CD5 + CD7 + cells emerged within the first 14 days on the DL4-expressing stroma, followed by CD4/CD8 double positive T cells by day 28, and CD3 + TCR$\alpha\beta$ or TCR$\gamma\delta$ cells by day 42. Genomic DNA sequencing showed that these cells had undergone TCR rearrangement [87]. In 2014, two other papers reported the generation of T cells with broad TCR diversity. One study used both hESCs and skin biopsy–derived iPSCs as the starting material. Here, PSCs were initially plated onto OP9 cells for 18 days to generate cell clumps containing CD34 + cells, which were then affinity-purified and replated onto OP9-DL4 with FL, SCF, and IL-7 for up to an additional 35 days. $\gamma\delta$T cells were first to emerge after 14 days on OP9-DL4, followed within a week by $\alpha\beta$ T cells, thus mimicking in vivo T-cell development. In vitro characterization confirmed that stimulation with PMA/ionomycin or CD3/CD28 beads induced expected activation responses in these T cells. They upregulated CD25 and CD69 expression and increased production of IL-2, IFNγ, TNFα, as well as the degranulating enzymes perforin and granzyme B [88]. Another study in 2014 avoided the use of OP9 cells for initial hematopoietic differentiation of hESCs as well as fibroblast- or BM-derived iPSCs. They demonstrated that tenascin C is a critical hematopoietic-inducing factor secreted by OP9 which mimics the extracellular matrix of in vivo HSC niches. In their study, PSCs were initially differentiated on a tenascin C substrate for 9 days, at which time CD43 + cells were replated onto OP9-DL4 to induce lymphoid lineage differentiation. Within 3 weeks, CD4 + /CD8 + double positive T cells emerged and showed evidence of polyclonal VDJ recombination [89].

During the early 2010s, efforts were underway to determine if iPSCs could be made from PB cells instead of reprograming dermal fibroblasts back to a pluripotent state. Not only is blood cell collection less invasive than a skin biopsy but the number of cells that can be collected is relatively high. Moreover, the use of blood cells for reprograming into iPSCs and then differentiating back again into blood cells may be beneficial due to epigenetic memory of the starting material and, in the case of T cells, can make use of preexisting TCR rearrangements and antigen specificity of the starting T cells. Toward that end, Loh et al. showed in 2010 that PBMCs could be reprogramed to iPSCs and more specifically, that mature T cells within the blood could give rise to iPSC clones [90]. This observation was followed by studies showing that antigen-specific, isolated, cytotoxic T lymphocytes (CTLs) could be reprogramed to iPSCs, expanded, and then redifferentiated back into CTLs while preserving the TCR repertoire and antigen specificity of the source material. For example, Nishimura et al. reprogramed antigen-specific CD8 + T cells from an HIV patient back into iPSCs. These were initially differentiated for 15 days on CH3H10T1/2 stroma with VEGF, SCF, and FL to give rise to CD34 + cells, which were then transferred onto OP9-DL1 in the presence of FL and IL-7 for up to an additional 30 days to differentiate into T cells. Stimulation of these developing T cells with CD3/CD28 beads or PHA followed by coculture with irradiated PBMCs plus IL-7 and IL-15 pushed them to generate memory CD8 + cells that were capable of mounting a cytotoxic response upon exposure to a specific HIV antigen. The stimulation/coculture step also enabled a $\sim 100-1000\times$ expansion of the iPSC-T cells as compared to parental T-cell clones under this condition, which were only able to expand $20\times$ [91]. The authors noted that these cells had elongated telomeres compared to the initial T-cell clone, helping one to explain their greater proliferative capacity. Another study demonstrated the use of melanoma epitope MART-1-specific CTLs to generate iPSCs. Coculture of the differentiating iPSCs on OP9-DL1 gave rise to CD4 + CD8 + double positive T cells expressing TCRβ, with roughly 70% being positive for MART-1 specificity, as evidenced by tetramer staining. Stimulation of these cells with anti-CD3 for 6 days induced a $300\times$ expansion of CD8 + and increased the percentage of MART-1-specific cells to >95%. Subsequent exposure to MART-1-expressing antigen-presenting cells (APCs) induced them to secrete IFNγ, as expected [92].

Encouraged by the recent clinical success of CAR engineering in primary T cells, CAR technology is now also being applied to PSC-derived lymphocytes. The generation of CAR-expressing PSC-derived lymphocytes could provide a readily available, highly effective therapy in situations where isolated T cells have not expanded well for patients or who do not respond well to donor lymphocytes. In a 2013 study by Themeli et al., PB T cells were first reprogramed into iPSCs using retrovirus and then subjected to lentiviral transduction of a second generation CAR construct, 19−28z. The 19−28z CAR construct has antigen specificity for CD19 and a dual CD28, CD3ζ internal signaling domain. These CAR-containing iPSCs were differentiated to EBs for 10 days, whereby the resulting CD34 + cells were isolated and transferred to OP9-DL1 coculture containing SCF, FL, and IL-7. After 30−35 days, mRNA profiling indicated that the resulting T cells were most similar to primary γδ T cells, despite their premature expression of TCRαβ. Exposure to CD19-expressing 3T3 cells resulted in upregulation of CD25 and CD69 as well as secretion of TNFα, IL-2, and IFNγ. Exposure to CD19-3T3 cells was also leveraged to induce their expansion, such that 3 weekly stimulations enabled a $1000\times$ expansion. The expanded T cells were able to mount CD19-specific cytotoxic responses against CD19-expressing EL-4 and Raji cells in a manner similar to that of primary γδ T cells as shown in an in vitro chromium release assay and an in vivo xenogenic tumor model, respectively [93]. The expression of CARs in PSC-derived T lymphocytes has great therapeutic potential, and we are likely to see further development of such therapies in the future.

Lymphocytes—NK cells

NK cells are a component of the innate immune system that accounts for $\sim 5\%-15\%$ of circulating lymphocytes. They can also be found in peripheral tissues such as the thymus, lymph nodes, spleen, liver, and lung [94,95]. NK cells circulate for roughly 2−4 weeks during which time they are able to provide rapid, nonspecific defense against various microbial infections and aid in the detection and elimination of tumor cells [96]. Cytokine release, granule-mediated natural cytotoxicity, target-cell apoptosis via membrane-bound Fas ligand and TRAIL expression, and antibody-dependent cellular cytotoxicity (ADCC) are the main mechanisms by which NK cells provide this defense. A balance between inhibitory and activating signals are required for NK cells to elicit each specific function. For example, the NK activating receptor NKG2D is involved in surveying MHC I−associated proteins and activating the NK cell if it determines that there is insufficient MHC I expression. Similarly, the activating receptor DNAM-1 is involved in the detection of stress signals such as CD112 and CD155 on the surface of tumor cells or infected cells to help trigger NK cell activation. Other activating receptors include natural cytotoxicity receptors such as NKp30, p44, p46, and heterodimers CD94/NKG2C, CD94/NKG2E as well as CD16, which

recognizes the Fc portion of antibodies and is involved in mediating ADCC. The CD94/NKG2A heterodimer is a major inhibitory receptor, while various types of killer cell-immunoglobulin like receptors (KIRs) are also present on the surface and can be either activating or, in most cases, inhibitory. In general, CD56 bright, CD16 low, KIR low, CD94 − immature NK cells are the principal cytokine secretors and reside mostly in lymph nodes while CD56 dim, CD16 high, KIR high CD94 + mature NK cells are largely responsible for cytotoxic responses and are found in the circulation [95,97].

Groundbreaking work in the 1980s by Rosenberg et al. stimulated considerable interest in and a variety of approaches for harnessing the cytotoxic capabilities of NK cells [98,99]. Autologous NK therapies are currently used in the clinic to help treat a variety of cancers including metastatic melanoma, renal cell carcinoma, and hematologic malignancies. In 2004, allogeneic NK cells were first tested for clinical use as an adjunct immunotherapy after HSCT for myeloid malignancies [100]. Since then, a variety of studies have confirmed that allogeneic NK cells are safe to use and therapeutically beneficial in the context of both combination therapies and monotherapies for various hematologic malignancies, Ewing sarcoma, breast and ovarian cancer, melanoma, renal carcinoma, and non-small cell lung cancer (reviewed in Ref. [101]). Interestingly, evidence suggests that allogeneic NK cells may be more effective than autologous ones, in part, perhaps because they avoid inhibitory signals from self-MHC receptors on autologous cells [102]. Currently, there are hundreds of ongoing clinical studies, sponsored by both academic groups and biotechnology companies such as Glycostem, Green Cross Lab Cell, and NantKwest to further explore the therapeutic utility of allogeneic NK cells from umbilical CB, unrelated PBMC donors, and the cell line, NK-92 (clinicaltrials.gov). Moreover, additional efforts are focused on enhancing the cytotoxic functionality of NK cells, improving their ex vivo expansion, and priming them before being adoptively transferred. For example, Genentech and Kyowa Hakko Kirin are conducting clinical trials to test monoclonal antibodies with enhanced affinity for CD16 in an effort to facilitate NK ADCC. AvidBiotics and Affimed are conducting preclinical and clinical studies using bispecific antibodies to recruit NK cells to tumor sites, and Innate Pharma is running clinical studies to test antibodies that bind to and block inhibitory receptors on an NK-cell surface [101]. Efforts to improve the expansion capability of NK cells are also being explored; membrane-bound IL-21 and nicotinamide have each been found to enhance ex vivo NK proliferation [103−105]. Preexposure of NK cells to cellular lysates from leukemia cell lines or to peptides from tumor-associated hsp70 (before they are infused into patients) are both being tested in clinical trials as potential ways to prime NK cells for greater in vivo efficacy [106,107].

A notable recent advancement for NK-based therapeutics is the adoption of CAR technology. First, second, and third generation CARs have all been engineered within NK cells or NK cell lines to target various cancer-associated antigens. Examples include targeting of CD19 and CD20 on leukemia and lymphoma cells, GD2 on glioblastoma, CS1 or CD138 on multiple myeloma, ErbB2 on breast, ovarian, and squamous cell carcinoma and CD5 on T-cell leukemias (reviewed in Refs. [108,109]). Second generation CARs, with multiple intracellular signaling domains, appear to work better than first generation CARs in NK cells [110] and 4-1BB (from CD137) appears to work better than CD28 as a costimulatory intracellular signaling domain [111]. CAR-NK cells may also have therapeutic advantages over CAR T cells due to their greater diversity of activating enzymes, various cytotoxic mechanisms of action, and shorter half-life [112]. Indeed, CAR-NKs can be designed to overexpress their natural activating receptors for improved cytotoxic effects rather than being designed to only target a single antigen. This is particularly advantageous in situations when a tumor has downregulated antigen expression and CAR T cells directed toward that antigen begin to elicit on-target but off-tumor effects. Chang et al. retrovirally introduced an NKG2D-DAP10-CD3z construct to enhance NK cell−mediated antitumor effects [113]. These engineered NK cells released a variety of cytokines and toxic enzymes through degranulation for improved cytotoxicity against a variety of cancer types in an antigen-independent manner. Another study reported that CAR NK cells were engineered with a TGFβ type II receptor fused to a transmembrane domain and intracellular signaling domain from NKG2D to allow them to be activated instead of inhibited by high TGFβ in the tumor microenvironment. These TGFβIIR-engineered NK cells migrated to TGFβ-producing tumors and increased IFNγ secretion for enhanced killing of hepatocellular carcinoma cells in a xenograft model [114]. CAR T cells secrete a panopoly of proinflammatory cytokines and their persistence in the circulation can lead to tissue-damaging CRS, yet CAR NK−secreted cytokines have less inflammatory potential and the shorter half-life of NK cells compared to T cells also helps limit the potential for CRS [112].

There are at least 10 ongoing clinical trials examining the therapeutic potential of CAR-NK cells in a variety of indications, including leukemias, B-cell lymphomas, ovarian, and prostate cancers (clinicaltrials.gov). NK sources include both primary NK cells, for example, from CB or the use of NK cell lines, such as NK-92, each of which has its own advantages and disadvantages. Primary NK cells express a wider range of activating receptors than NK cell lines and can somewhat expand in vivo in

response to activating signals. However, primary NK cells are limited by short supply, difficulties with in vitro expansion, and although they can be isolated from other cell types, pose the risk of contaminating T cells, which could elicit GvHD. NK cell lines, on the other hand, are homogenous and can easily be expanded to a large-scale in vitro but lack certain activating receptors such as CD16 and cannot expand in response to in vivo cues due to the need to irradiate them before use.

As these studies are ongoing, there has been considerable interest in developing NK cell therapies from PSCs as an alternative source that may combine the advantages of primary NK and NK cell lines. Leading the way, Kaufman et al. have published a series of papers showing proof of concept and optimization of differentiation protocols for the generation of functional hESC- and iPSC-derived NK cells. Their initial protocol used began with coculturing PSCs on murine M210-B4 stroma for 17−20 days, isolation of the resulting CD34 + CD45 + progenitors, which represented <5% of the culture, and transfer of these progenitors onto murine AFT024 stroma in media containing SCF, FL, IL-7, and IL-15. After a 30−35-day total, CD45 + CD56 + CD94 + NK cells emerged [115,116]. A variety of in vitro assays showed these cells secreted IFNγ in response to IL-12/IL-18 stimulation, displayed natural cytotoxicity and ADCC against various cancer cell lines. In vivo xenograft studies showed they had antitumor activity [115] as well as anti-HIV activity [117]. This group has made several improvements to their protocol to make them more clinically relevant. They are now able to avoid the use of the M210-B4 stroma step by instead using an 11-day spin EB step to generate CD34 + CD45 + progenitors [118]. They subsequently transfer the entire spin EB culture (not just the isolated progenitors) to uncoated 24-well plates in the presence of IL-3, IL-7, IL-15, SCF, and FL instead of onto ADT024 stroma. They noted that the contents of the spin EB culture contain cells that attach to the 24-well plate to provide its own stromal layer. Within 4 weeks, the culture produces CD45 + CD56 + CD94 + NK cells expressing a variety of other NK markers that are comparable to those generated in their original protocol. Moreover, these cells similarly can be induced to secrete IFNγ, display natural cytotoxicity and ADCC against a variety of cancer cell types [118]. The group was also able to use a K562-based, membrane-bound IL-21 expressing artificial APC line to help expand their PSC-NK cells 2 to 3 logs. These various improvements to PSC-NK differentiation protocols have resulted in clinical realization of the technology. In November 2018, Fate Therapeutics announced that it had received FDA approval to begin a Phase 1 clinical trial (NCT3841110) testing iPSC-derived NK cells for the treatment of advanced solid tumors as a monotherapy or in combination with immune checkpoint inhibitors [119].

In parallel, Kaufman's group has continued to advance their platform by applying CAR technology to their PSC-NK cells [120]. In a recent study, they screened nine different CAR constructs containing an scFv with specificity for the tumor-associated antigen, mesothelin and various options for cotransmembrane and costimulatory domains in NK92 cells to determine which may be most efficacious in their PSC-NK cells. The screen indicated that a novel construct with the transmembrane domain of NGK2D, 2B4 costimulatory domain (from CD244), and the CD3ζ signaling domain, which is also a domain expressed within CD16, worked best in an NK-cell background. They engineered iPSCs to carry this CAR and compared functionality of the resulting meso-specific NK CAR iPSC-NK cells to nonengineered iPSC-NK, T cells, meso-directed CD28−41BBz (CAR-T) expressing iPSC-CAR NK cells as well as meso-directed CAR T cells. They found their iPSC-CAR-NK cells were more effective in inhibiting in vivo ovarian cancer cell growth and prolonging survival of the tumor xenograft mice than the regular iPSC-NK cells, T cells, and CD28−41BBz (CAR-T) expressing iPSC-CAR NK cells. The antitumor effect was comparable to that of CAR-T cells, yet the NK-CAR iPSC-NK therapy provided longer term survival of the xenografted animals than the CAR T cells, likely due to a shorter cytokine release period and thus cytokine-induced toxicity than the CAR T cells [120]. These results suggest that the specific domains used to generate a CAR should be matched to the cell in which it is expressed for optimal therapeutic function.

Lymphocytes—NKT cells

NKT cells are another lymphocyte population with therapeutic potential and for which both PSC and CAR technologies are being applied. They represent ≤1% of circulating lymphocytes and have characteristics of both T cells, via expression of TCRαβ, and of NK cells, via expression of CD161 (NK1.1), CD16, and CD56. Class I NKT cells express an invariant α chain on their TCR, the Vα24 Jα18, thus accounting for the term iNKT ("invariant" NKT). They are further defined as being either CD4 + , CD8 + , or CD4/CD8 double negative. iNKT recognize lipids that are presented through CD1d on target cells, such as alpha-galactosylceramide (a-GalCer) and can be induced to secrete various cytokines to activate other immune cell populations, including CD8 + T cells, NK, and DC cells [121]. They also can produce degranulating toxic enzymes and use Fas-ligand- and TRAIL-mediated methods to induce apoptosis in target cells. Class II NKT cells are also CD1d-restricted but do not recognize a-GalCer. They have greater diversity in their TCR and recognize a wider range of antigens and are thought to be largely immunosuppressive [122]. Clinical

interest in NKT cells have therefore largely focused on class I, the iNKT cells. iNKT are able to infiltrate solid tumors better than T cells and contribute to antitumor responses by killing tumor-associated macrophages and myeloid-derived suppressor cells that normally contribute to tumor growth and immune resistance within tumor microenvironment. Evidence also suggests that they may help suppress GvHD (reviewed in Ref. [123]). Preclinical studies have suggested potential utility of primary iNKT in treating cancers ranging from leukemia and lymphoma to melanoma and colon, breast, ovarian, prostate, and lung cancers. Clinical studies have likewise tried to leverage the antitumor properties of iNKT. Approaches include direct injection of a-GalCer or injection of ex vivo, a-GalCer-pulsed CD1d-expressing myeloid-derived DCs to augment endogenous iNKT activity. Adoptive transfer of ex vivo expanded iNKT cells is also being tested as a monotherapy or in a combination with a-GalCer-pulsed PBMC to treat nonsmall cell lung cancer, head and neck cancer, melanoma and multiple myeloma (reviewed in Ref. [123]). CAR technology is also being applied to primary NKT cells. Heczey et al. showed that first, second, and third generation CARs, when expressed in NKTs, could mediate antigen-specific lysis of neuroblastoma cells and increased the survival of tumor-bearing mice, while the CAR NKT cells still retained their inherent ability to kill CD1d + tumor-associated macrophages [124]. This has led to the first wave of clinical trials testing CAR-NKT technology. A Phase 1 clinical trial for autologous GD2-specific, IL-15-expressing CAR-NKT is being conducted for neuroblastoma (NCT03294954) while another Phase 1 study using allogeneic CD19-directed CAR NKT cells is being conducted for relapsed or refractory B-cell lymphomas (NCT03774654).

Given the low numbers of primary NKT in circulation, several groups have tried to find ways to increase yields for off-the-shelf ACT. A recently reported approach involves treatment of allogeneic NKT with IL-2 and IL-21, which was found to preserve the central-memory-like phenotype and antitumor effects of the long-lived CD62L + NKT cells [125,126]. Another approach involves the use of PSCs as an alternative starting material for adoptive iNKT cell therapies. Watarai et al. developed an NKT differentiation protocol that was successfully used to generate NKT cells from both mouse ESC and iPSCs [127,128]. In their first study, mouse ESCs generated through nuclear transfer from an NKT cell were used to differentiate into NKT with the hope that the prearranged invariant TCRα gene from the NKT nucleus would allow the ESCs to differentiate better than standard ESCs. These NKT-ESC were cultured on OP9-expressing DLL1 in the presence of FL for 10 days to induce their differentiation. Suspension cells were then transferred to fresh OP9-DLL1 stroma plus FL and IL-7 every 4 days thereafter. Analysis of the various fractions showed that Notch signaling via DLL1 is important for early stage NKT development but that functional maturation out to day 20 was not impacted by DLL1. These cells helped generate antigen-specific, IFNγ-secreting CD8 + T cells in vivo. The same protocol was used to generate NKT from iPSC and these were also found to be functional as they secreted large amounts of IFNγ and reduced tumor growth in vivo. Yamada et al. adapted this OP9-DLL1-based protocol for the differentiation of human iPSCs. These iPSCs had originally been reprogramed from PB or CB Vα24 + iNKT and redifferentiation of the resulting iPSCs back into iNKT was achieved within 33 days. The newly derived iNKT were found to be Th1-skewed in nature, which lends them to therapeutic use; they showed better cytolytic activity against six different tumor lines than a reference NKT cell line. They also displayed antitumor effects using a K562-luciferase tumor model and could be expanded 4−10 × within one to two additional weeks via stimulation with a cocktail containing IL-2, IL-7, and IL-15 [129]. Another study the same year adapted a previously published T-cell differentiation protocol [91] to generate iNKT from an iNKT-derived iPSC line. They cultured the iPSCs onto C3H10T1/2 stroma in the presence of VEGF for 7 days, upon which time they added SCF and FL to induce hematopoietic differentiation. At 14 days, the hematopoietic progenitors were collected, transferred to OP9-DLL1 stroma in media with supplemental IL-7 and FL and on day 29, they added IL-2 and IL-15. The resulting iNKT-iPSC-iNKT-harbored characteristic NKT properties: they secreted IFNγ in response to a-GalCer stimulation, induced maturation of DCs and their IL-12p70 secretion, activated T and NK cells, and displayed NK-like cytotoxicity against cancer cell lines [130]. Additional studies are underway to continue developing the therapeutic potential and clinical application of PSC-NKT.

Monocyte-derived dendritic cells

Straddling the interface between innate and adaptive immunity, DCs are one of the body's three main types of APCs. Clinically, DCs may be used in the development of vaccine-based therapies to stimulate T-cell responses against a variety of disease-associated antigens [131]. Upon exposure to a suitable antigen, immature DCs undergo the process of activation and maturation, which involves proteolyzing antigen and presenting its fragments on the DC-cell surface using MHC class I or II molecules for CD8 + or CD4 + T cells to recognize, respectively. Myeloid-derived DCs arise from monocytes, secrete IL-12 in response to activating stimuli, and express toll-like receptors TLR2 and TLR4. Lymphoid-lineage-derived DCs [plasmacytoid (p)DCs] have similar functional

characteristics to mDCs but secrete IFNα and express TLR7 and TLR9 [132]. Specialized DCs called Langerhans cells are located in the skin and mucosa, while DCs can also be differentiated from CD34 + CB cells. Innovative work in the late 1990s provided the proof of concept for clinical use of DCs as studies showed that ex vivo generated DCs (from allogeneic or autologous BM or PB sources) could be loaded with melanoma-specific antigens and stimulate antitumor immune responses once injected into patients [133,134]. Since then, many different types and sources of DCs have been investigated for their utility in developing cancer vaccines. Currently, there are over 400 ongoing clinical trials testing DC-based vaccines as a monotherapy or in combination with other approaches (clinicaltrials.gov). A wide range of diseases are being targeted with monocyte-derived DC-based vaccines, including leukemia, lymphoma, melanoma, multiple myeloma, HIV, hepatitis C, glioblastoma, hepatocellular carcinoma, colorectal, ovarian, prostate cancer, and many more. The design of such studies varies considerably as DCs may be loaded in vivo or ex vivo with either a single antigen, antigenic peptide, whole killed tumor cells, tumor cell lysate, apoptotic bodies, exosomes, or tumor-derived DNA or RNA [135].

Several studies have shown that human PSCs can differentiate into DCs and may serve as a cost-effective and scalable standardized source of DCs. Two main differentiation procedures have been used to generate DCs from hESCs and iPSCs. The first method relies on OP9 coculture [136,137], while the second utilizes an EB-based approach and can be done in a serum-free or serum- and feeder-free manner [138–140]. The resulting DCs in these studies had characteristic large eccentric nuclei, spiny dendritic processes, and expressed DC surface markers, CD11c, CD40, CD45, CD86, HLA class I and II to varying degrees with two to five DCs per starting PSC being generated. Despite subtle differences compared to monocyte-derived DCs, PSC-derived DCs produced in these studies and in our own lab appear to be functional upon maturation in assays measuring IL12-p70 secretion, chemotaxis, antigen-uptake and proteolysis, induction of T-cell proliferation and stimulation of antigen-specific cytotoxic CD8 + T-cell responses ([138,140] and unpublished results). In addition, the discovery of CD141 + tolerogenic DCs in the skin [141] has led to an exploration of PSCs to generate DCs capable of resetting tissue homeostasis. Sachamitr used a 24-day DC differentiation protocol coupled with negative selection for CD1c to isolate a tolerogenic CD141 + DC population that could be used to induce tolerance to local antigens and reset homeostasis in autoimmune diseases such as type 1 diabetes [142]. Further development of such PSC-derived DCs is still needed before they may compete with donor-derived DCs.

Monocyte-derived macrophages

Macrophages are large phagocytic leukocytes that play a role in normal tissue homeostasis and local immune responses. Populations of tissue-specific macrophages are established during normal fetal development and can survive for months to years [143]. However, in response to chemotactic signals, circulating monocytes extravasate from blood vessels differentiate into macrophages and adopt tissue-specific gene expression and functions. Examples of diverse tissue-resident macrophages include liver Kupffer cells, central nervous system microglia, and lung alveolar macrophages. Macrophages engulf and digest microbes, cancer cells, and foreign substances as an innate immune defense mechanism and assist in the activation of adaptive immune responses. Interest in harnessing the defensive properties of macrophages dates back to the 1970s when Dr. Isaiah Fidler began testing the antitumor effects of isolated macrophages in mouse tumor models [144]. Subsequently, several clinical trials conducted during 1987–2010 aimed to use autologous macrophages to treat cancers, including nonsmall cell lung cancer, bladder, colorectal, and ovarian cancers, pancreatic cancer, and renal cell carcinoma (reviewed in Ref. [144]). Although such trials showed that large and frequent doses of autologous macrophages are safe and well tolerated, most had little, if any, therapeutic benefit. Recent research has revealed the complexities of macrophage polarization, optimized culture conditions, and the value of engineering for directing macrophage function in vivo, which has renewed interest in developing macrophage-based therapies for clinical use. Toward that end, the company Vericel has developed Ixmyelocel-T, an autologous BM-derived mixed cell therapy enriched for M2 antiinflammatory macrophages and MSCs to treat dilated cardiomyopathy and critical limb ischemia. In 2016 results from their Phase 2b trial for ischemic heart failure showed a 37% reduction in cardiac events for patients treated with Ixmyelocel-T [145], and in 2017 the company received FDA's Regenerative Medicine Advanced Therapy designation to facilitate expedited review of their macrophage/MSC therapy for ischemic heart failure [146]. Likewise, skewing macrophages toward an M1 subtype is thought to promote their antitumor effects [147,148]. This knowledge, combined with various engineering approaches, has provided greater control over the functions and in vivo effects of macrophage-based therapies in development. For example, a recent study shows that expressing a HER2-targeting CAR in macrophages could significantly inhibit tumor growth in a HER2-4T1 orthotopic tumor model in BALB/C mice. These CAR-macrophages helped degrade tumor extracellular matrix and enabled greater T cell infiltration into the solid tumor [149]. Abbvie has also invested in a

CAR-macrophage startup company called Carisma to target solid tumors, such as those expressing Her-2 and two other undisclosed targets [150], while various other engineered macrophage-based therapies are in preclinical development [144].

Protocols for the differentiation of PSCs into monocytes and macrophages have been developed and refined over the years. Wilgenburg et al. have described a clinically adaptable, chemically defined, serum- and feeder-free method for the generation of CD14 + CD16 low CD163 + monocytes from both hESC and iPSCs. Application of M-CSF further differentiates them into phagocytic macrophages with properties similar to those of primary macrophages [151]. Additional studies have applied genetic engineering approaches to more effectively control the in vivo effects of PSC-derived macrophages. For example, Koba et al. genetically engineered an iPSC-derived myeloid/macrophage cell line to overexpress IFNβ to treat NUGC-4 human gastric tumors, noting that the overexpression of IFNβ was essential for an antitumor effect [152]. In another example, Senju et al. engineered iPSCs with an scFv directed toward beta amyloid and showed that iPSC-derived macrophages could specifically phagocytose this Alzheimer's disease−associated protein in vitro. They also generated another iPSC line with an scFv directed against CD20 and differentiated them into macrophages cells, demonstrating these CD20-specific iPSC-derived macrophages could specifically kill BALL-1 B-cell leukemia cells both in vitro and in immunocompromised mice [137]. In follow-up studies, this group engineered iPSC macrophages to express the beta amyloid-degrading enzyme, neprilysin, which reduced soluble beta-amyloid in vivo [153]. In another example, gene editing has been performed in iPSCs from patients with hereditary pulmonary alveolar proteinosis (PAP) to correct mutation of the CSF2RA gene that is required for alveolar macrophages to properly clear surfactant protein [154−156]. Pulmonary transplantation of these gene corrected iPSC-macrophages functionally compensated for defective macrophages in a humanized mouse model of PAP [154]. These are just a few examples of how combinations of PSC and gene editing and/or CAR technologies are being applied to myeloid-lineage leukocytes. Their continued development holds great promise for future novel regenerative medicine-based therapies.

Granulocytes—neutrophils

Granulocytes are myeloid-lineage WBCs that help fight pathogenic infections and play a role in allergic reactions by mediating inflammatory responses. They harbor abundant granules in their cytoplasm containing a variety of toxic enzymes that can be released in response to environmental signals. Four main granulocyte classes (neutrophils, eosinophils, basophils, and mast cells) have varying roles in fighting bacterial, viral, and parasitic infections. They can be characterized by the distinct shape of their nuclei and hematoxylin−eosin staining patterns. Neutrophils are the most abundant leukocytes in the PB and account for 60%−70% of circulating WBCs with a concentration of roughly $2.5-7.5 \times 10^9$/L. They rapidly kill microbes, cancer cells, and other foreign entities through phagocytosis and the production of reactive oxygen species. With a half-life of 6−8 hours, neutrophils are abundantly produced on a magnitude of 5×10^{10} to 10×10^{10}/day [157].

Neutropenia describes the situation when absolute neutrophil counts are less than 1500 cells/μL and severe neutropenia occurs when counts are below 500 cells/μL. Neutropenia may be caused by chemotherapy or as a result of primary immunodeficiency, such as chronic granulomatous disease (CGD) and Kostmann syndrome [158]. Evidence suggests that rates of infection increase with increasing severity of neutropenia. Roughly, a third of severely neutropenic patients will develop rapid, life-threatening infection within a single week [159]. G-CSF (Filgrastim), pegylated G-CSG (Pegfilgrastim, which has a longer half-life), or a recently FDA-approved biosimilar (Zarxio) can be used to stimulate the production of neutrophils and represents the main therapeutic approach to treating neutropenia. Allogeneic granulocyte transplantation (Gtx) can also be used to provide rapid, short-term relief from critical neutropenia, yet the collection of procedure is time-consuming and limited by the short half-life and shelf-life of mature neutrophils. In the United States, Gtx has largely been replaced by Filgrastim and antibiotic/antifungal therapies [160]. In certain chronic situations, long-term G-CSF/Filgrastim cannot be used and HSCT is the only curative therapy. In more acute situations, such as following chemotherapy, there is still a gap where patients are at increased risk of infection even when given Filgrastim and antibiotic/antifungal medications [161]. In these situations, there is a need to increase the absolute neutrophil count through other means. Toward this end, nicotinamide-expanded CB stem cells (NiCord) have been found to shorten the time to neutrophil engraftment, reduced the incidence of severe infections, and reduced the duration of hospitalizations within the first 100 days following treatment. Their contribution to short-term recovery is likely due to the expansion of myeloid progenitors that can quickly differentiate into neutrophils for faster recovery than unmanipulated HSCs alone [162,163].

The propensity of PSCs to differentiate into myeloid progenitors and/or further differentiate down the neutrophil lineage has been explored for similar therapeutic purposes. In 2009, hESC-derived CD11b + neutrophils were

generated through an EB method and were found to be functional in three in vitro assays assessing chemotaxis, phagocytosis, and production of reactive oxygen species [164,165]. In one of these studies the hESC-neutrophils displayed IL-1b-induced chemotaxis in an air-pouch inflammatory mouse model [165]. In subsequent studies, Morishima et al. used an OP9 coculture method to generate three characteristic subsets, containing either azurophilic, lactoferrin, or MMP9-containing granules [166]. These neutrophils expressed characteristic neutrophil transcription factors, C/EBPα and ε, could phagocytose, respond to chemotaxic signals, and displayed bactericidal activity. In another study in 2016, Sweeney et al. used a four-stage, 32-day EB-based protocol to generate and expand hematopoietic stem and progenitors and then induce their differentiation into granule-expressing neutrophils with similar morphology and function to primary neutrophils [167]. Similarly in 2017, Schrimpf et al. differentiated marmoset (*Callithrix jacchus*, "cj") iPSCs into CD34 + /VEGFR2 − hematopoietic progenitors and then further into neutrophils. These cj-iPSC-derived neutrophils showed similar cell surface markers, subcellular structures, and phagocytic properties to primary neutrophils in a variety of in vitro tests and will facilitate preclinical evaluation of the technology in nonhuman primates [168].

The earlier-mentioned studies have laid down the foundation for the development of gene-edited PSC-based approaches to treat primary immunodeficiencies. In CGD, mutations in the gene for CYBA, CYBB, NCF-1, NCF-2, and NCF-4 cause disruptions in NADPH oxidase, which is needed for the production of superoxide and the antimicrobial functions of phagocytic leukocytes such as neutrophils. Patient-specific iPSCs from individuals suffering from CGD have been subjected to zinc-finger nuclease and TALENS-based editing to correct the underlying genetic mutations in X-linked CGD at the CYBB locus or through insertion of a corrective minigene at the AAV1 safe-harbor locus. Subsequent differentiation of the corrected iPSCs into neutrophils has shown the functional recovery of neutrophil activity [169−172]. Transplantation of gene-corrected iPSC derivatives offer a regenerative medicine approach to functionally compensate for defective granulopoiesis in CGD patients in the short or perhaps even long term.

Perspectives

As reviewed here, regeneration and replacement of PB components can be used to treat a variety of diseases and indications. PSCs have a limitless ability to self-renew and as such have garnered significant interest as an inexhaustible cell source for the generation of off-the-shelf myeloid and lymphoid lineage blood cells. PSC-derived RBCs and MKs/platelets may one day be used in transfusions to treat anemia and thrombocytopenias, yet further development is still needed to mature these cells and/or produce them on an exponentially larger scale. PSC-derived DCs can be developed as off-the-shelf cancer vaccines as an alternative to the primary DC-derived cancer vaccines currently being tested in more than 400 clinical trials. The success of CAR technology in primary lymphocytes has sparked interest in developing PSC-derived T, NK, NKT cells, and macrophages with a diverse array of CARs to treat hematologic malignancies, various solid tumors, Alzheimer's, and other diseases. In addition, PSCs can be used to generate neutrophils or their progenitors and may provide bridging therapies to help fight infections in patients undergoing myeloablative chemotherapy and HSCT or suffering from severe neutropenia due to other causes. Moreover, when combined with gene-editing technology, iPSCs can be used to create patient-specific gene-corrected neutrophils, macrophages, or their progenitors, thus offering a way to treat primary immunodeficiencies that impair the functions of these cells. Ultimately, PSC-based blood cell therapies have a unique opportunity to treat a wide variety of disorders and provide readily available, commercial-scale regenerative medicine to a large number of patients. Moreover, the combination of gene therapy, gene editing, and/or CAR technologies in PSCs makes them a powerful tool to drive the development of highly innovative, blood cell-specific cellular therapies.

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Chapter 43

Red blood cell substitutes

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Introduction

Blood type incompatibility reactions, short ex vivo storage shelf-life (42 days in the United States), and the risk of contracting unknown pathogens are some of the issues that have been associated with allogenic red blood cell (RBC) transfusion. To this end, researchers have long sought a RBC substitute that is universal, stable at ambient temperature and pressure, and nonpathogenic. Such a fluid would need to replicate the functionality of RBCs, while surpassing the aforementioned limitations associated with RBCs. Despite rigorous efforts to develop an effective RBC substitute in the late 20th and early 21st century, there is currently no clinically approved RBC substitute in either the United States or Europe. Regrettably, the inherent toxicity of RBC substitutes and their nonphysiologic performance obstructed the possible clinical applications. Complications of RBC substitutes include nonphysiologic O_2 delivery, vasoconstriction, systemic hypertension, and renal toxicity. Other strategies to produce these materials yielded safer materials but were too costly to pursue clinically. Current development of RBC substitutes now focuses heavily on design strategies that mitigate toxicity and restore physiologic performance. Other research has focused on developing materials that would make RBC substitutes closer in costs to an allogenic-packed RBC unit. Current RBC substitutes consist of either synthetic perfluorocarbons (PFCs) or a large variety of modified hemoglobin (Hb) species (Fig. 43.1) that can replicate various RBC functions.

Replicating red blood cell functions

In the circulatory system, RBCs perform a variety of functions (Fig. 43.2), including facilitating O_2 and carbon dioxide (CO_2) transport between the tissues and the lungs, Hb detoxification via encapsulation, and reduction via methemoglobin (metHb) reductase, and small molecule reductants, vascular flow modulation via shear-stress induced signaling [1–3], and nitrate reactions [4–6]. An ideal RBC substitute should have material properties that replicate each of these functions. Initially development of RBC substitutes only focused on O_2 transport. Recently developed materials have incorporated aspects of CO_2 transport and Hb detoxification. In addition to replicating the core functionality of RBCs, a practical RBC substitute must have reasonable circulatory half-life and be easily metabolized or excreted by the body. These materials must also meet or exceed the ex vivo shelf-life of a human blood unit to be applicable in a clinical setting. There are two classes of RBC substitutes that meet some of these functions: Hb-based O_2 carriers (HBOCs) that bind O_2 and synthetic PFCs that dissolve O_2.

Hemoglobin-based oxygen carriers

HBOCs represent the first major class of RBC substitutes. These O_2 carriers are derived from Hb, a 64.5 kDa metalloprotein found in RBCs that is responsible for O_2 storage and transport. Hb is composed of two $\alpha\beta$ dimers, with each α and β subunit holding a single heme prosthetic group in a hydrophobic binding pocket. The iron in the core of the heme prosthetic group is capable of reversibly binding O_2. In the lungs ($pO_2 \sim 144$ mmHg), Hb contained inside RBCs is typically 98% saturated with O_2 (1.34 mL O_2/g of Hb). When the RBCs reach the tissues ($pO_2 \sim 20–40$ mmHg) the Hb inside the RBC discharges most of its O_2. This ability to nonlinearly offload O_2 results from intramolecular interactions between O_2, heme, and the globin subunits. The release of O_2 triggers a conformational change from the high affinity oxygenated relaxed (R) quaternary state to the low-affinity deoxygenated tense (T) quaternary state (Fig. 43.3). During the transition between the T- and R-state, the structure of each globin subunit shifts conformation, which results in changes in the ion pairs at the α-β globin interface [7].

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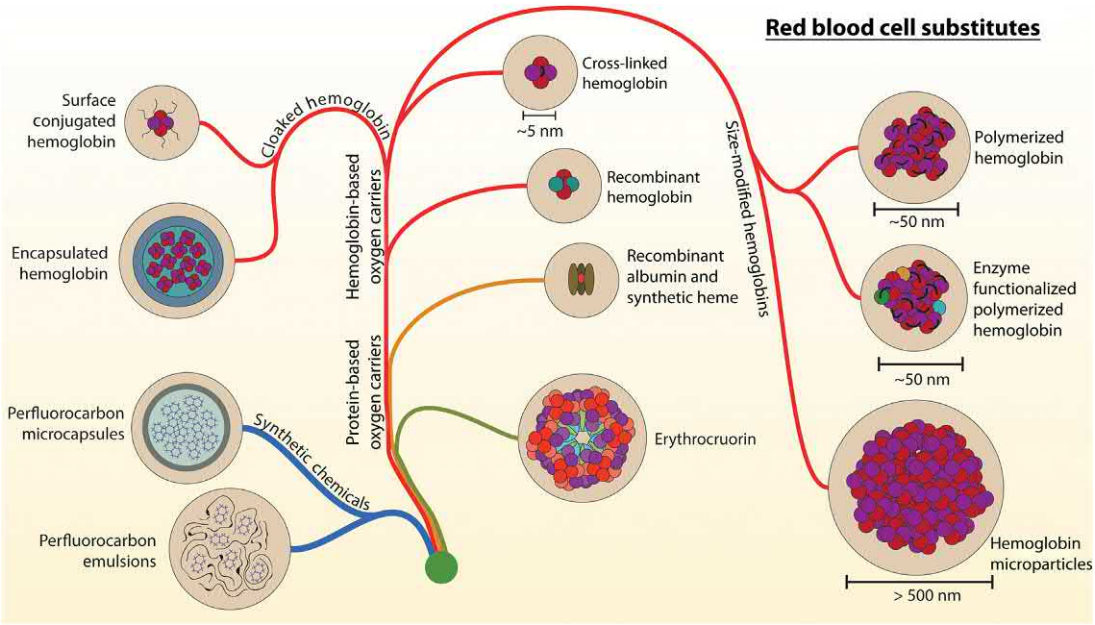

FIGURE 43.1 Common types of red blood cell substitutes.

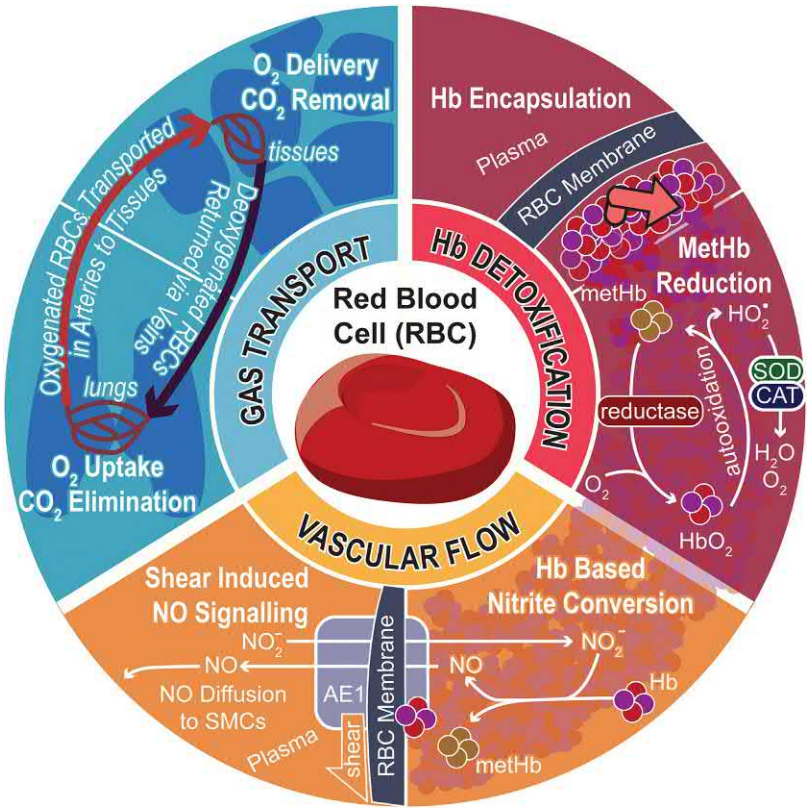

FIGURE 43.2 Functions of a red blood cell.

These conformational shifts lead to cooperative O_2 binding that facilitate O_2 offloading at physiologic conditions. This nonlinear O_2 binding is captured in the signature sigmoidal O_2 equilibrium curve of Hb. In addition to O_2, diphosphoglycerate (2−3 DPG) in RBCs can bind to deoxyhemoglobin (deoxyHb) to stabilize the tense quaternary state which decreases the O_2 affinity of Hb. Another allosteric effector is carbon dioxide (CO_2). This gaseous

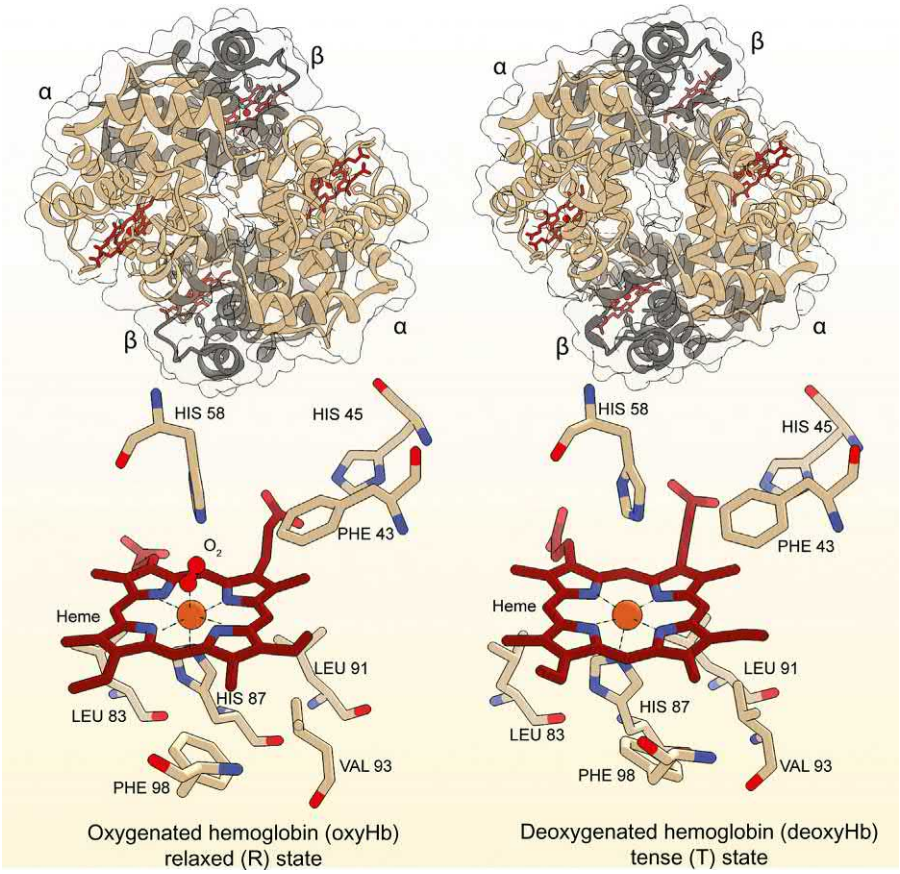

FIGURE 43.3 Human hemoglobin tetramers in the T and R quaternary states with a view of the α subunit heme binding pocket in the corresponding state. Molecular graphics images were produced using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH P41 RR-01081). *R*, Relaxed; *T*, tense.

ligand binds as a carbamate to the four terminal-amine groups of the protein subunits in T-state Hb further altering the O_2 affinity. This binding function also results in Hb storing and transporting 20% of the CO_2 in blood (4 mol CO_2/mol deoxyHb). Because the core functionality of the RBC (O_2 transport) depends on Hb, this protein is typically considered a key component in the formulation of many HBOCs. By isolating Hb from RBCs, blood group antigens are removed from HBOCs which eliminates crossmatching requirements and permits rapid administration. In addition, HBOCs can be stored for several years either as a dried powder or a deoxygenated solution at room temperature for use in emergency scenarios.

Hemoglobin toxicity

While purifying hemoglobin from RBCs reduces the risk of transmission of unknown pathogens, increases ex vivo storage lifetime, and eliminates blood type incompatibility reactions, administration of stroma-free Hb in the circulation is associated with renal toxicity and hypertension [8]. The mechanisms of Hb toxicity are well understood, and several mechanisms for toxicity have been proposed. The iron containing heme prosthetic group that is responsible for Hb's ability to bind O_2 is the primary component associated with the toxicity of acellular Hb. Hb can oxidize from the ferrous ($HbFe^{2+}$) form to the ferric ($HbFe^{3+}$) form known as metHb which is incapable of binding O_2. When inside RBCs, metHb accumulation is limited and protected by enzymes such as metHb reductase, catalase (CAT), and superoxide dismutase (SOD) [9]. Outside the RBC, Hb is exposed to exogenous oxidants that accelerate metHb formation. Furthermore, metHb is prone to αβ dimerization followed by the release of heme [10]. These ferric species can spontaneously form ferryl (4 + valence state) species in aqueous environments, which leads to the production of reactive oxidative species (ROS) such as superoxide and hydrogen peroxide ions. These ROS result in oxidation of lipids, protein, DNA, and mitochondria. Furthermore, the ROS produced during autoxidation drive a catalytic pseudoperoxidase cycle that oxidizes Hb to metHb and ferrylHb ($HbFe^{4+}$) [8]. The ferrylHb and its associated radical formed during the pseudoperoxidase cycle have high redox potentials (1.0 V) that further aggravate oxidative stress [11]. In the absence of allosteric effectors, most HBOCs have higher redox potentials than unmodified Hb. Despite this, most HBOCs have

increased rates of autoxidation that has been attributed to increased heme accessibility [12].

Furthermore, when in the ferric state, the iron in the heme prosthetic group no longer forms a covalent bond with the proximal histidine residue (αH87, βH92) in the hydrophobic heme binding pocket. The absence of the covalent bond results in the release of free heme that can accumulate in tissues. Of concern is the accumulation of free heme within cell membranes and the subsequent activation of Toll-like receptors which amplifies inflammatory responses [13]. To combat these effects, plasma proteins such as haptoglobin and hemopexin reduce tissue oxidation/ROS generation and aid in free Hb/heme clearance, respectively [14,15]. Unfortunately, these mechanisms are quickly exhausted due to the volume of HBOC transfusions that are typically required for emergencies.

One of the key factors linked to Hb toxicity is the protein's small molecular diameter. With a hydrodynamic diameter estimated at only 5.5 nm [16] as a tetramer (and less when dissociated into αβ dimers), Hb can freely extravasate through the vascular endothelium and into the interstitial space and tissue space [17]. Moreover, when acellular Hb extravasates through the blood vessel wall, it can inhibit NO signaling from endothelial cells to smooth muscle cells, which results in vasoconstriction and systemic hypertension [18].

The pathway for this inhibition has been well studied. In the interstitial space, HbO_2 rapidly ($\sim 10^7\ M^{-1}/s$) and irreversibly reacts with NO produced by the vascular endothelial cells to yield metHb and nitrate, effectively blocking NO uptake by the smooth muscle cells. Without the endogenously produced NO, proendothelin is converted to endothelin, which stimulates contraction of the smooth muscle cells and subsequent vasoconstriction [19]. This increased vasoconstriction then led to increased pulmonary hypertension and reduced cardiac output. In addition, the increased diffusion and subsequent endothelial interactions between nanoscale HBOCs may lead to trace NO scavenging at the endothelial wall [20].

Tissue extravasation of these acellular Hbs and the transfusion volume can lead to significant accumulation of Hb and heme within the kidney [21]. An early clinical study that attempted to transfuse acellular Hb led to rapid clearance and renal dysfunction [22]. Organ failure typically occurs due to ROS production from the accumulated Hb in the kidney. A key aspect of modern HBOC design is the mitigation of Hb's oxidative potential and vasoactivity via encapsulation, size modification, or the addition of antioxidant activity (Fig. 43.4). However, reigning in the inherent toxicity of Hb is extremely difficult once Hb is outside of the protective barrier of the RBC. Many of the approaches employed can only partially reduce the toxicity of HBOCs.

Increasing the size of HBOCs to reduce extravasation is the principal method to reduce the toxicity of acellular Hbs. Studies have found that the hydrodynamic diameter of HBOCs are inversely proportional to their vasoactivity and oxidative toxicity. Because of this, the design of many HBOCs aim to increase the stability of the Hb tetramer and the HBOC diameter [23,24]. Increasing HBOC size also increases the circulatory half-life of the materials by reducing clearance in the liver, kidney, and spleen [25] and potentially by obstructing the haptoglobin clearance pathway [26]. Many strategies to increase the size of Hb have been investigated, including polymerization, surface conjugation, and encapsulation.

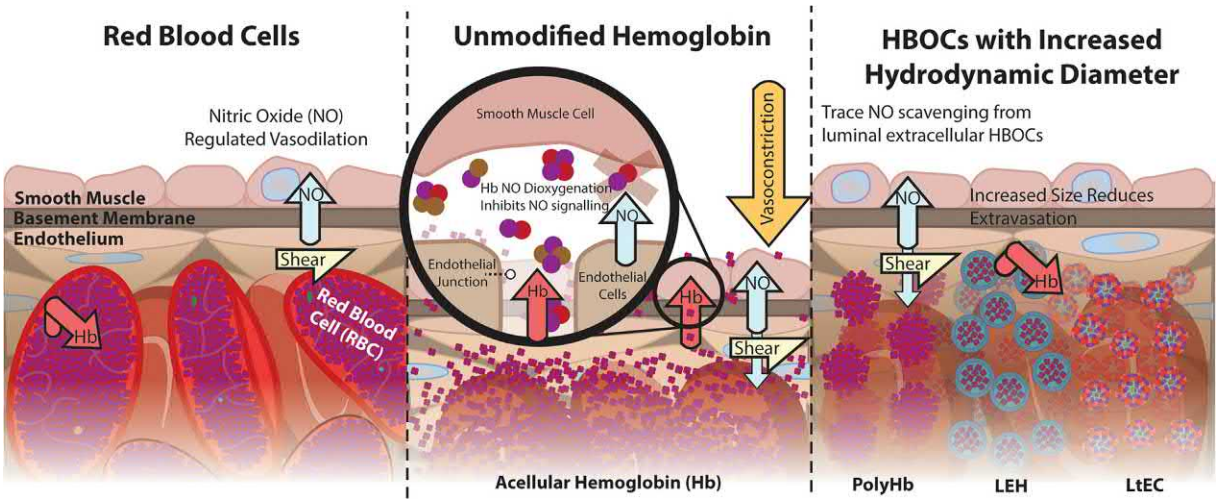

FIGURE 43.4 The role of Hb extravasation on inhibition of NO signaling from the endothelium and smooth muscle cells. HBOCs with increased hydrodynamic diameter do not extravasate, with only minimal scavenging from luminal HBOCs due to decreased contact with the endothelial vessel wall. *Hb*, Hemoglobin; *HBOCs*, hemoglobin-based oxygen carriers.

Oxygen delivery

In addition to tissue extravasation and oxidative tissue injury, the nonphysiologic O_2 offloading of many early generation HBOCs is suspected to correlate with either increased hypertension or unsuitable oxygenation. The various modifications that are employed to reduce toxicity and increase circulatory half-life alter the O_2 affinity of Hb (Fig. 43.5). In general, these modifications either increase or decrease the O_2 affinity of Hb. In some cases, where the protein conformational motion is limited by intermolecular cross-links, the cooperativity is also eliminated. Low O_2 affinity HBOCs are typically formed when HBOCs are produced under anoxic conditions ($pO_2 \sim 0$ mmHg). These species can have as little as 60% of the O_2 binding capacity of unmodified Hb at atmospheric pressure. Because of their low O_2 affinity, these HBOCs readily offload O_2 but require higher Hb or O_2 concentrations to bind an equivalent amount of O_2 compared to the Hb inside RBCs. In addition, these low O_2 affinity species may offload O_2 while in the arterioles leading to arteriolar overoxygenation autoregulatory effects [27]. High affinity HBOCs are typically produced when reacting Hb under hyperoxic conditions ($pO_2 \sim 140-700$ mmHg). In addition, other modifications can separate the Hb into $\alpha\beta$ dimers, which have characteristically high O_2 affinity like myoglobin. In contrast to low affinity HBOCs, high O_2 affinity HBOCs do not offload O_2 in arterioles and are fully saturated when in the lungs. Instead, these species retain O_2, only releasing it under ischemic or hypoxic conditions. There is still ongoing debate in the development of artificial RBCs whether low or high O_2 affinity HBOCs are more effective. Some studies have also attempted to mix low and high O_2 affinity HBOCs to establish intermediate O_2 affinities [28]. In addition, many of the chemical modifications used to prepare HBOCs eliminate or reduce the Bohr effect [26].

The potential reduction of O_2 affinity could also lead to an oversupply of O_2 that may lead to vasoconstriction. In the absence of the intraluminal diffusive barrier of the lipid bilayer present on the RBC surface, O_2 diffusion is enhanced due to the extracellular Hb species that can freely diffuse throughout the intravascular space [29,30]. This process of facilitated diffusion can lead to arteriolar vascular constriction and decreased functional capillary density due to excessive O_2 offloading to the arterioles [31,32].

Viscosity and colloid osmotic pressure

In addition to altering O_2 delivery, transfusion of a large volume of HBOC leads to altered flow in the vascular system. Transfusion of RBC substitutes reduces the hematocrit and thus apparent blood viscosity via hemodilution. Hemodilution increases blood flow velocity but decreases blood vessel wall shear stress that is necessary for mechanotransduction and subsequent regulation of functional capillary density via NO production in the endothelium [33,34]. The resultant supraperfusion increases the O_2 carrying capacity of blood, which in turn decreases transit losses to nonvital tissues and increases O_2 delivery within the capillary network [35]. Unfortunately, many of the chemical modifications used to decrease the toxicity and improve performance of HBOCs alter their viscosity and colloid osmotic pressure (COP). However, some of the strategies employed to increase HBOC size, such as Hb polymerization, can also lead to significant increases in viscosity, which impairs heart function and leads to high blood pressure. To match the COP of blood, RBC substitutes typically aim to have COPs between 20 and 25 mmHg. Application of fluids with low COP, such as saline and lactated Ringers, leads to tissue edema due to increased fluid transport into the tissue space. In contrast,

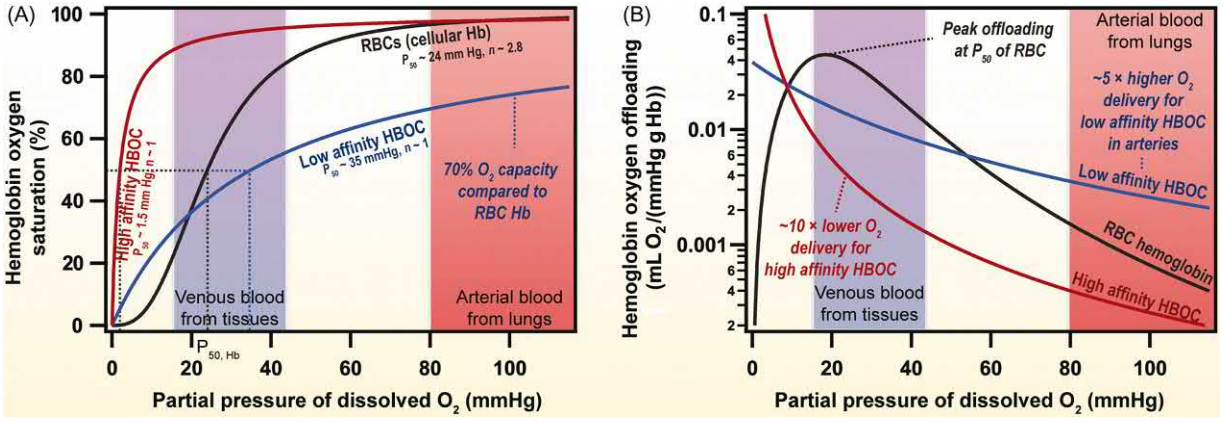

FIGURE 43.5 OEC and oxygen offloading plot for Hb inside RBCs, a low O_2 affinity HBOC, and a high O_2 affinity HBOC. *Hb*, Hemoglobin; *HBOC*, hemoglobin-based oxygen carriers; *OEC*, oxygen equilibrium curve; *RBC*, red blood cell.

transfusion of solutions with high COP leads to fluid transport from the tissue into the vasculature which can hinder nutrient delivery. However, high COP may be beneficial for reversing endothelial edema during hemorrhage [36]. To maintain the O_2 carrying capacity of blood, most HBOCs must be formulated at a concentration of at least 10 g/dL. Unfortunately, the chemical modifications that reduce toxicity typically increase the viscosity and/or COP that are directly proportional to the concentration of the HBOC. For example, poly(ethylene glycol) (PEG) surface conjugated Hb (PEG−Hb) species have extremely high COP (50 mmHg) at moderate concentrations (4 g/dL) [37]. This limits the overall O_2 carrying capacity of these solutions. Higher concentrations of PEG−Hb have increased COP which leads to fluid transport from the tissue space into the vascular space which dilutes the blood volume and further decreases blood O_2 carrying capacity.

Cross-linked and polymeric hemoglobin

As discussed previously, modifying the size and increasing the stability of stroma-free Hb can significantly decrease toxicity. One of the simplest methods to increase the size of the HBOC is through reacting the surface amino acid side chains (lysine, valine, etc.) of Hb with cross-linking reagents such as glutaraldehyde, *O*-raffinose, bis(3,5-dibromosalicy)-adipate, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. These reagents serve to form covalent bonds between the protein subunits, which increase the overall size of the HBOC. This has traditionally been one of the more popular methods to formulate HBOCs with many materials, including Oxyglobin, Hemopure, Polyheme, Hemolink, and Oxyvita, that have underwent clinical trials in the United States and Europe. Oxyglobin and Hemopure (OPK Biotech, Cambridge, MA) are moderate molecular weight (200−250 kDa) glutaraldehyde polymerized Hbs that had success in Phases I and II clinical trials. PolyHeme is a glutaraldehyde polymerized pyridoxal phosphate cross-linked Hb product developed by Northfield Laboratories (Evanston, IL) [38]. Despite being approved for veterinary use in the United States, Oxyglobin consists of 31% unpolymerized Hb that led to vasoconstriction, hypertension, oxidative stress, and iron deposition in endothelial, neural, and renal tissues [39−41]. Hemopure only has 2% unpolymerized Hb that overall resulted in reduced hypertension [42,43]. However, the low molecular weight of these materials led to negative vascular side effects in patients undergoing aortic and orthopedic surgery [44,45].

Instead of using glutaraldehyde as a cross-linker, Hemosol Inc (Toronto, ON, Canada) used *O*-raffinose to cross-link Hb to form the polymerized Hb product Hemolink. This material showed reduced NO affinity and hypertension in early animal studies in rats. The production process led to a heterogenous distribution of molecular weights which showed significant vasoactivity in Phases II and III clinical trials on coronary artery surgery patients. Hemolink had a low molecular weight (150 kDa), which likely led to organ failure in rats and vasoconstriction in lambs [46,47]. Despite this, production and subsequent clinical trials were abandoned due to ethical concerns during the final clinical trial [48]. One of the reasons these early materials demonstrated poor performance is the overall low molecular weight of the molecules. However, increasing the molecular weight of polymerized Hb significantly decreases hypertension and oxidative tissue injury [25].

To combat vasoconstriction, hypertension, and oxidative stress, OXYVITA Inc. (New Windsor, NY) developed a high molecular weight (17 MDa) HBOC known as OxiVita [49]. This HBOC was instead produced with cross-linking the beta subunit with bis(3,5-dibromosalicy)-adipate combined with tetramer polymerization using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [50,51].

In addition, other enzymes such as SOD, CAT, and carbonic anhydrase (CAN) can be cross-linked into the polymeric Hb structure to mitigate oxidation and increase CO_2 solubility [52−54]. Cross-linking these antioxidant species into the polymerized Hb structure reduced hypertension. Additional studies have conjugated fibrinogen to the polymerized Hb complex to facilitate coagulation in addition to oxygenation [55]. Because increasing the size of the polymeric Hbs has a significant effect on the side effects induced by cell-free Hb, some researchers have aimed to make Hb nano- and microparticles. One such material is manufactured via the coprecipitation of Hb into a manganese carbonate matrix with glutaraldehyde surface polymerization [56−58]. These particles can have narrow size distributions with negative zeta potentials. Unfortunately, the enormous size of some of these Hb microparticles (>700 nm) can increase phagocytosis and will most likely lead to macrophage activation if the size is not suitably decreased [59]. Despite numerous chemical modifications, many of these cross-linked and polymerized Hb species still had relatively short circulatory half-lives ($\sim$24−48 hours).

Surface conjugated hemoglobin

To increase HBOC circulatory half-life, Hb can be surface conjugated with PEG to cloak the Hb from the immune system. PEG, benzene tetracarboxylate dextran, hydroxyethyl starch, and albumin have all been conjugated to the surface of Hb to increase its circulatory half-life. The PEG conjugated Hb, MP4OX (MP4, Hemospan), has undergone extensive clinical testing. This is a high O_2 affinity Hb species with low cooperativity and Bohr effect that has been generally determined to be safe and

effective [60]. However, these species have been shown to scavenge NO at the same rate as many other HBOCs [18]. Their high COP (50 mmHg at 4 g/dL) limits its concentration in the circulation. Furthermore, its high O_2 affinity and similarity to PEG surfaced conjugated albumin indicate that it acts as a plasma expander [61]. Some studies have shown that steric interactions between the PEG groups conjugated to the Hb tetramer led to $\alpha\beta$ dimerization and high O_2 affinity [26]. More recently, SynZyme Technologies LLC (Irvine, CA) developed a polynitroxylated $\alpha-\alpha$ cross-linked Hb (VitalHeme). This product was designed to take advantage of the O_2 transport of Hb, plasma expansion of PEG, and antioxidant behavior from polynitroxylation [62]. Other studies have attempted to cloak the Hb in a human serum albumin (HSA) core shell structure via covalent bonds [63]. While surface conjugation increased circulatory half-life, it did not always increase the size enough to prevent extravasation into the tissue space and hypertension.

Encapsulated hemoglobin

To both increase HBOC hydrodynamic diameter and vascular retention, researchers can also encapsulate Hb inside vesicles to form artificial cells. Encapsulation of Hb inside vesicles composed of lipids or amphiphilic polymers presents a close analogue to the structure of RBCs. In these HBOCs, Hb is encapsulated inside the aqueous core of vesicles that prevent Hb from directly interacting with the vasculature. Apart from reducing interaction with the vasculature, encapsulation can also reduce facilitated diffusion associated with cell-free Hb. The high Hb concentration inside the vesicle also stabilizes Hb in its tetrameric form. During production of these nanoparticles, it is possible to coencapsulate antioxidants, allosteric coeffectors, and other components to mimic the RBC cytoplasm more closely.

Early iterations of these materials used lipid bilayers to encapsulate Hb inside liposomes. These materials demonstrated low circulatory half-life due to aggregation and liposome fusion during storage [64]. To increase circulatory half-life, reduce plasma component interaction, and increase storage stability, PEG was preferentially incorporated into the outer surface of the vesicles to form PEG conjugated liposome encapsulated Hb (PEG−LEH) [65]. Further studies found that increasing the length of PEG chains increased the circulatory half-life of the PEG−LEH particle [66]. However, over increasing the molecular weight of PEG chains leads to the formation of PEG-lipid micelles [67] and complement activation suppression [68,69]. Like polymerized Hb, increasing the size of the PEG−LEH particle led to a decrease in vasoconstriction and hypertension at similar doses [24]. PEG−LEH particles are primarily cleared through the spleen and liver leading to increased lipid levels 1 week after transfusion [70] and abnormal splenic behavior that persists for several weeks [71]. A primary challenge with the production of LEH species is severe oxidation of the encapsulated Hb (50%−75% metHb) that occurs during encapsulation. Unfortunately, the purification process that isolates Hb from the RBC matrix also removes many of the RBC proteins that are responsible for the reduction of metHb. To combat Hb autoxidation, many LEH products are saturated with carbon monoxide (CO) to stabilize Hb in the Fe^{2+} state. The strong bond between CO and the iron in the heme ligand not only eliminated autoxidation but also the O_2 carrying capacity due to competitive inhibition. Another strategy to reduce autoxidation of Hb consists of coadministering methylene blue and its metabolic byproducts as an electron transfer mediator between LEH and the Embden−Meyerhof−Parnas and pentose phosphate pathways in RBC cytosol [72,73].

Another concern with PEG−LEH species is the low serum-half-life resulting from shear degradation. An actin matrix can be introduced into the core of LEH to increase the mechanical stability of the LEH that further increases half-life [74]. Another alternative is encapsulating Hb within a PEG−polylactic acid membrane nanocapsule with polymerized Hb, SOD, CAT, and CAN. Because of the addition of these components, these materials had reduced phagocytosis and decreased metHb levels [75]. Another alternative is the encapsulation of Hb in nonbiodegradable biocompatible hydrophilic lipogels. These lipogels can be photopolymerized within the lipid bilayer to give hybrid properties of the hydrogels and liposomes [76]. An alternative encapsulation technique uses amphiphilic diblock copolymers (polymersomes) that contain a hydrophilic (PEG) and hydrophobic block to form vesicles [77−79]. These materials allow for control of the membrane thickness and PEG corona [80]. These materials also showed reduced vasoactivity and NO dioxygenation compared to PEG−LEH. Unfortunately, the process of polymersome formation yields extremely low Hb encapsulation efficiency ($\ll 10$ g/dL) which limits the O_2 carrying capacity. Because of the low O_2 carrying capacity, PEG−LEH remains the most attractive type of encapsulated HBOC.

Sources of hemoglobin

The primary source of human-derived Hb is derived from freshly expired human-packed RBC units. Because removing Hb from RBCs eliminates blood group antigens, Hb can be also be derived from nonhuman sources. Many of these nonhuman HBOCs derive their Hb from blood waste from the meat-processing industry. Because of this, HBOCs have incorporated bovine and porcine Hb as potential source materials. Bypassing the need for human

Hb by using bovine Hb for HBOC production can help with patients who refuse allogenic blood transfusions due to religious grounds [81]. However, substantial portions of the global population are unable to receive transfusions originating from bovine, porcine, or other animal sources due to religious or moral beliefs. An alternative to these methods is the production of recombinant Hb using bacterial or yeast cultures to produce Hb.

Recombinant hemoglobin

Development of recombinant Hb began in 1980s with the eventual expression of HbA in transgenic pigs [82], *Escherichia coli* [83], and *Saccharomyces cerevisiae* [84]. Initial yields were modest with 24% HbA in transgenic pigs and 2%−10% expression in *E. coli* and *S. cerevisiae* [85]. Unfortunately, this form of Hb is still prone to the toxicity associated with acellular Hb species. To aid with mitigating the inherent toxicity of recombinant Hb, researchers began to explore genetic engineering to improve its function outside RBCs. The key goals were to decrease NO reactivity, modulate O_2 offloading, decrease autoxidation, and mitigate heme release [85].

The primary method of engineering recombinant Hb is based on rational mutagenesis to decrease toxicity and increase O_2 delivery. One such modification is the use of the di-α gene with a single cysteine link between the C-terminal end of one α subunit and the N-terminal of the second α subunit (rHb0.1) [86]. Because of the di-α link, rHb0.1 does not dissociate into αβ dimers under physiologic conditions. Because of this increase in stability, rHb0.1 serves as a basis for many recombinant HBOCs. The first of these, which underwent preclinical and Phase I clinical trials, was expressed in *E. coli* with glycine-fused α subunits and the Presbyterian mutation (N108K) to decrease O_2 affinity to match that of RBCs (rHb1.1) [85,86]. In a small animal study, rHb1.1 was highly vasoactive because of the small size of the molecule and unmodified NO scavenging potential [87,88].

Following rHb1.1 Baxter Therapeutics and the Olsen group developed an rHb (rHb3011) with glycine fused α subunits, a mutation to reduce capture volume in the distal portion of the heme pocket (αL29W, βV57W) to decrease NO scavenging, and a mutation that weakens that stabilization of bound O_2 to heme (αH58Q) that assists with O_2 dissociation [19,89]. To further reduce extravasation and increase circulatory the half-life, rHb3011 was polymerized and surface conjugated with PEG to form rHb2.0. This material significantly reduced vasoconstriction and hypertension compared to rHb1.1 [87]. Unfortunately, the αL29W mutation that lowered NO reactivity also decreased Hb stability and increased autoxidation and heme loss [85]. Furthermore, compliment activation was observed in clinical trials resulting from autoxidation, heme loss, and subsequent protein degradation [90].

Apart from the mutations used in rHb0.1, rHb1.1, and rHb2.0, other mutations have been considered, including mutations to increase apohemoglobin stability (βG16A, βH116I, αG15A) [91], decrease O_2 affinity by allosterically stabilizing the T-state of the tetramer (αV96W, βK82D) [92,93], and decrease heme dissociation (βS44H) [94]. Other researchers have also proposed replacing tyrosine and methionine residues with phenylalanine to decrease the lifetime of protein radicals to decrease metHb formation and increase Hb stability [95]. Some researchers have proposed that directed evolution and random mutagenesis could be used to screen for Hb with more desirable properties. Despite advancements in directed evolution, screening the resulting large libraries of Hb is limited by protein purification and biophysical analysis [85].

A key concern in recombinant Hb-based O_2 carrier (rHBOC) production is the prohibitive cost of recombinant protein expression combined with low yields. To this end, rHBOC development has recently focused on methods to reduce the cost per gram of rHBOC produced. A major limiting factor is that endogenous heme ligand group production by *E. coli* limits the formation of Hb. This can be solved via addition of the first molecule involved in the porphyrin synthesis pathway and δ-aminolevulinic acid [85]. However, the high cost of δ-aminolevulinic acid can limit production. Instead, excess exogenous hemin is added so that hemin can be introduced via passive diffusion into the cell. Unfortunately, the addition of hemin leads to hematin species that must be removed via compilated purification processes. Researchers have also developed coexpressed heterologous heme transport systems such as plasmids for heme utilization genes (pHUG) [96] and plasmids for hemoprotein expression (pHPEX) [97] to decrease the total amount of heme required. Alternatively, some researchers are using *E. coli* cell lines with higher porosity (BL-21) [98] to reduce the amount of heme required. Because most systems still rely on bacterial expression, additional purification steps to remove lipopolysaccharides and other endotoxins bind to Hb to prevent pyrogenic responses [99]. An alternative may be to use heterologous expression in mammalian systems [100]. As an extension on the molecular engineering of rHBOCs, recombinant albumin has been genetically engineered to conjugate to synthetic tetraphenylporphinatoiron (II) complexes. This complex is capable of reversibly binding O_2 with low O_2 affinity [101,102].

Erythrocruorins

Each of these earlier HBOC design strategies aimed to modify Hb to reduce the toxicity that results from the small molecular diameter of Hb. Thus an alternative

design strategy would be to use an Hb source derived from animals that lack RBCs and have thus evolved Hb-like proteins that can exist outside of the protective environment of RBCs. The earthworm (*Lumbricus terrestris*) is one such animal. *L. terrestris* erythrocruorin (LtEc) is the O_2 carrying protein found in earthworms. This massive 3.6 MDa molecular weight protein contains 144 heme containing globin subunits and 36 nonheme linker proteins held together with disulfide bonds and protein interactions to form a 30 nm diameter nanoparticle [103,104]. LtEc is also less prone to oxidation into the Fe^{3+} and Fe^{4+} states due to the lower solvent exposure of the heme binding pocket and higher redox potential (+112 mV) compared to HbA (−50 mV) [105–107]. Initial applications in small animal models demonstrated that this protein was able to delivery O_2 without causing systemic hypertension [108–110]. In fact, the moderate COP (14 mmHg) and negligible rate of NO scavenging likely led to vasodilation in animal models. Recent work has sought to improve LtEc circulatory half-life and stability via PEGylation, glutaraldehyde cross-linking, and poly(acrylic acid) conjugation [111–113].

The erythrocruorin of the lugworm, *Arenicola marina* erythrocruorin (AmEc) is another annelid Hb that has a much lower rate of autoxidation ($-0.005\ hour^{-1}$) compared to HbA ($0.014\ hour^{-1}$) [106]. In addition, AmEc has a much lower NO scavenging rate compared to HbA [114]. Because of these properties, it is currently being developed as HEMOXYCarrier (Hemarina SA) with positive results in small animal studies. Apart from AmEc the erythrocruorins of several ocean worms including *Oligobrachia mashikoi* and *Glossoscolex paulistus* are strong RBC substitute candidates due to their extreme heat tolerance and hydrogen sulfide transport characteristics [115,116]. However, each of these marine worm species have typically evolved to optimize O_2 transport under subnormothermic conditions (4°C–8°C), thus many of them are not well suited for O_2 delivery under physiologic conditions (37°C) in humans. For both LtEc and AmEc a key concern is their supply. Unlike Hb sourced from humans, cattle, swine, or other mammals, erythrocruorins have limited commercial availability and require extensive purification for use as an RBC substitute [117].

Perfluorocarbons

The second class of RBC substitute is PFCs. PFCs are biologically inert synthetic chemicals composed entirely of carbon and fluorine atoms that are capable of transporting O_2 and other gaseous ligands. These O_2 carriers can exist as either straight or cyclic hydrocarbon chains with the general chemical formula of C_nF_{2n+2}. Common PFCs used in the development of artificial blood include perfluorodecalin, perfluorooctyl bromide, and perfluorotripropylamine. Because of the strong chemical bonds between the fluorine and the carbon atoms, the body is incapable of metabolizing PFCs. These strong bonds also make PFCs highly hydrophobic and lipophobic [118]. Unlike Hb, PFCs do not bind O_2. Instead, the high solubility of O_2 in PFC solutions can increase the total concentration of O_2 in the plasma. For example, the PFC perflubron dissolves 0.697 μL O_2/(mmHg mL PFC) (59.5 μL O_2/mL PFC under atmospheric conditions) [119]. Unfortunately, because of the high insolubility of PFCs, emulsifiers such as albumin and egg yolk phospholipids must be added to solubilize PFCs. These emulsifiers limit the overall concentration of the PFCs that in turn necessitates greater doses to maintain systemic O_2 delivery. The first PFC emulsion approved by the FDA, Fluosol-DA only has an O_2 carrying capacity of 0.07 μL O_2/(mmHg mL PFC) (10.8 μL O_2/mL PFC under atmospheric conditions) which is much less than the O_2 carrying capacity of Hb in RBCs (450 μL O_2/mL blood) [120]. More recently developed PFC emulsions such as Oxygent have increased the O_2 solubility to 0.17 μL O_2/(mmHg mL PFC) (25.5 μL O_2/mL PFC under atmospheric conditions) [121]. Unfortunately, these materials still are not able to offload the same amount of O_2 compared to RBCs. The key difference between HBOCs and PFCs is the linear saturation of PFCs with O_2 as a function of increasing dissolved O_2 concentration. For example, a pure perflubron solution requires an atmospheric O_2 content of ~290 mmHg to match the O_2 delivering capacity of RBCs. In order to match the O_2 delivery capacity of RBCs, most emulsified PFCs require hyperbaric O_2 treatment to maintain the O_2 content in the lungs above 760 mmHg. Another alternative would be to increase the O_2 solubility of the PFC. To match RBC performance an ideal PFC emulsion would have to possess an O_2 solubility of ~1.2 μL O_2/(mmHg mL PFC). Unfortunately, this approximately doubles the solubility of pure PFC solutions and does not come close to approaching the O_2 carrying capacity of RBCs (Fig. 43.6).

Fortunately, PFCs are heat stable in excess of 300°C, which makes them amenable to heat-sterilization techniques. Because PFCs are composed of synthetic chemicals, PFCs can be transfused to individuals that refuse human blood or proteins derived from humans and/or animals. Despite early clinical success, Fluosol-DA was eventually pulled from the market in 1994 due to decreases in platelet count and flu-like symptoms resulting from macrophage activation due to the large size of the PFC particle droplets [122]. Following Fluosol-DA, many strategies were employed to improve emulsified PFC performance while decreasing negative side effects. OxyFluor (HemaGen, Missouri) is a perfluorodichlorooctane emulsion that had an overall higher O_2 carrying capacity compared to Fluosol. Despite its success in clinical trials, flu-like symptoms and thrombocytopenia halted development [123].

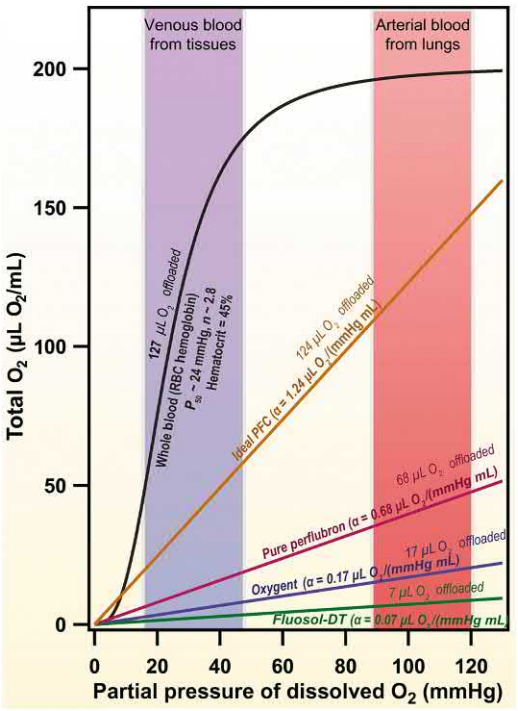

FIGURE 43.6 Total O_2 available from whole blood with a hematocrit of 45%, an ideal oxygen carrying PFC, a pure solution of perflubron, a pure emulsion of Oxygent, and a pure solution of Fluosol as a function of the partial pressure of dissolved O_2. The linear relations were calculated from the solubility of each PFC. O_2 offloading was calculated from the predicted O_2 delivered from the arterial (120 mmHg) to venous (20 mmHg) conditions. *PFC*, Perfluorocarbon.

Another PFC emulsion, Oxycyte (Synthetic Blood International, California), composed of perfluorodecane emulsified in egg-white phospholipid was tested in patients with traumatic brain injury in Phase II clinical trials [124]. However, additional Phase II trials were suspended for this material. Perftoran (Perftoran, Russia) was a perfluorodecalin and perfluoro-*N*-(4-methylcyclohexyl) piperidine emulsified in Proxanol-268 that was used in a variety of applications in Russia and Mexico [125]. Oxygent (Alliance Corp, California) is a perflubron/*F*-decyl bromide PFC emulsified with egg yolk phospholipid developed by Alliance Pharmaceutical Corp. Although Oxygent performed well in a Phase III general surgery trial [126], subsequent trials in cardiopulmonary bypass surgery led to adverse neurological events such as stroke [127]. However, researchers have speculated that these events were likely caused by a failure in the design of the clinical trial rather than from the material properties of the PFC emulsion.

One challenge associated with each of these PFC emulsions is the tendency of the particles to form large droplets. These large droplets elicit the production of cytokines by activated macrophages and impair neutrophil functions [122]. To combat large droplet formation, encapsulation of PFCs can be used to increase the solubility while maintaining a small droplet size. These methods can use synthetic polymers such as poly(lactic-*co*-glycolic acid)−PEG [128], biologic materials such as HSA [129], or RBC membrane fragments [130] to encapsulate the PFC droplets.

Perspectives

Despite decades of work, there is no clinically approved RBC substitute. However, recent work on the effects of HBOC size optimization and advances in reducing the cost of manufacturing and processing has reinvigorated corporate interest in HBOC development. One limiting factor associated with HBOCs is the cost to manufacture the materials. An ideal RBC substitute would ideally be cost-completive with a packed RBC unit. Unfortunately, many HBOCs require either expensive reagents, (PEGylated/encapsulated HBOCs), have low yields (recombinant HBOCs), or extensive purification (polymerized HBOCs/erythrocruorin) which increases the cost of a unit compared to a unit of RBCs. Recently, cost-effective large-scale purification methods such as tangential flow filtration have been used to help reduce manufacturing costs [110,131,132]. Lately, there has also been a tendency to combine several material modifications, such as PEGylation of LtEc [111] and rHb3011 [87], to increase overall performance and decrease side effects. PFCs are typically easy to produce and show no issues with NO scavenging and ROS generation. Furthermore, the introduction of improved emulsification/encapsulation methods in PFC preparation may help mitigate macrophage activation and improve stability. Despite these advantages, PFCs are still limited by the absence of physiologically relevant O_2 offloading that has been implicated with stroke and autoregulatory responses. In addition, despite approval for clinical applications, PFCs have issues associated with the large required doses and increased risk of stroke. Because no RBC substitute is capable of replicating all the functions of RBCs, RBC substitutes have primarily been applied as an "oxygen bridge" until normal blood transfusions can be applied in "extreme, life-threatening situations" [8]. Many companies and researchers are now looking past trauma, hemorrhagic shock, and anemia to other applications including cancer treatment, organ transplant preservation, and tissue-engineered construct oxygenation.

Organ transplant preservation

Due to their increased ability to dissolve O_2 versus aqueous solutions, PFCs have long been used for static cold organ preservation [133]. However, cold static preservation of organs can lead to progressive tissue damage, which directly effects organ function [134]. Machine perfusion of

organs is thought to mitigate the problems associated with cold static organ storage by providing O_2 and nutrients while removing metabolic waste products. Lately HBOCs and PFCs have also been applied to machine perfusion devices to increase O_2 delivery to the tissues. While RBCs may be added to the machine perfusion solution, there are still issues with hemolysis and subnormothermic RBC performance. Instead, both PFCs and HBOCs may be used as an oxygen carrying perfusate in organ perfusion circuits. PFC emulsions have been used to supplement organ perfusion solutions for canine, porcine, and human kidneys [135–137]. Preliminary studies with Hemopure for machine perfusion of porcine livers preserved hepatic function [134,135,138]. Another recent study used AmEc for subnormothermic perfusion of lung tissue that improved graft function [139].

Cancer treatment

Beginning in the early 1990s, researchers attempted to use RBC substitutes to make chemo and radiotherapy more effective in the treatment of cancer. These researchers tried a variety of materials, including polymerized Hb [140–145], PEG–Hb [146,147], and PEG–LEH [148–150]. In general, these studies have shown that HBOCs are effective at increasing O_2 delivery to the tumor tissue if large enough dosages are provided [151]. Supraperfusion was hypothesized to be one of the primary mediators of the increased O_2 delivery [152]. More recently computational models have predicted that the O_2 affinity of HBOCs is a primary determinant of O_2 delivery to hypoxic tissues [153,154]. In addition, some studies have found that PFCs, such as Fluosol-DA and Oxygent, can be used to facilitate increased O_2 delivery to tumors to increase the effect of radiotherapeutic, chemotherapeutic, and photodynamic treatment [155]. A recent article has even used ultrasound paired with PFCs to increase O_2 delivery directly to tumors [156].

Tissue-engineered construct oxygenation

A major barrier in tissue engineering is adequate O_2 supply to tissue scaffolds. Standard tissue-engineering methods rely on diffusive O_2 transport throughout the scaffold, which limits the size of the final construct. Oxygen is a vital for cellular respiration and differentiation [157,158]. Simply increasing the ambient O_2 supply can lead to ROS production and subsequent uncontrolled differentiation and inflammation [159–161]. HBOC supplemented media has been shown to increase aerobic respiration and drug metabolism to hepatocytes cultured within a hollow fiber bioreactor [162,163]. Mixing HBOCs with varying O_2 affinity may also be used to regulate tissue construct oxygenation [28]. In addition to HBOCs, PFC emulsions such as Oxygent and Oxycyte have been used to engineer cardiac muscles on a porous scaffold [164–166]. These PFC emulsions improved cell viability, function, and phenotype of the cultured cells.

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Part Thirteen

Kidney and genitourinary system

Chapter 44

Stem cells in kidney development and regeneration

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The kidneys are a pair of complex organs that are widely known for their role in urine production and waste elimination, but they also have numerous other responsibilities and are key regulators of normal physiologic processes, including electrolyte homeostasis, acid−base balance, fluid status, blood pressure control, maintenance of bone health, and erythropoiesis. These functions are executed by the collective activity of an array of individual units called nephrons, which are epithelial structures comprising a set of highly specialized segments. Renal failure develops when a significant number of nephrons are injured or lost, which results from a wide variety of acute and chronic etiologies in the clinical setting. Irreversible and progressive diminution of kidney function commonly leads to end-stage kidney disease necessitating renal replacement therapy (e.g., dialysis or transplantation), and this condition is increasing in worldwide prevalence. While dialysis is a reliable method to achieve fluid balance and solute clearance, it is associated with substantial morbidity and mortality, and it can only replace a small fraction of normal kidney functions. Renal transplantation, on the other hand, is significantly limited by donor organ availability and requires lifelong immunosuppression. As a result, there is an obvious need for novel approaches to the prevention and treatment of kidney disease, including tissue engineering and other potential regenerative therapies. One prominent strategy toward this objective is the use of stem cells to generate renal tissues, a field that has progressed considerably in recent years. These efforts have been grounded in an advanced understanding of the basic principles of kidney development, during which stem and progenitor cell types predominate. This chapter will provide a discussion of developmental mechanisms of renal embryology and stem cell biology, and we will review recent advances in regenerative technologies relating to the kidney.

Kidney development

Our current understanding of embryonic kidney development comes from decades of work in a variety of model organisms, including zebrafish, frog, chick, and a remarkable array of mouse genetic studies. The resulting principles have influenced nearly all subsequent efforts toward kidney regenerative medicine. Thus in this section we will review and summarize the relevant literature pertaining to kidney developmental biology, with a particular emphasis on the signaling pathways and tissue interactions that drive organogenesis. We also refer readers to other comprehensive reviews that more thoroughly describe the transcriptional regulators that coordinate nephrogenesis [1−3].

Throughout the course of evolution, the kidney has become increasingly complex and highly specialized. The metanephros is the latest and most sophisticated version of the organ, and it forms the functional adult kidney in amniotic organisms. A significant developmental advancement of the metanephros is an iterative branching process that leads to a compact organ comprising thousands to millions of individual nephrons. The preceding evolutionary structures, the pronephros and mesonephros, do not function in adult amniotes, although they fulfill the excretory needs of lower vertebrates and anamniotes. Importantly, they still form transient structures in mammalian embryos. These vestiges have essential embryologic roles, and their distinctive transcriptional networks and other cellular markers have helped direct developmental programs in vitro

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and guide our efforts to generate metanephric kidney tissue from stem cells.

Early embryonic origins of nephrogenic tissues

The kidney derives from a column of tissue, termed the intermediate mesoderm (IM), which is situated between the paraxial and lateral plate mesoderm (LPM). The IM, marked by expression of transcription factors Osr1 and Pax2 [4–6], extends from approximately the sixth somite to the caudal region of the embryo. The presumptive pro-, meso-, and metanephros are arranged in a collinear fashion along the anteroposterior axis. Early in development, a simple epithelial duct (termed the nephric or Wolffian duct) emerges from the anterior IM and begins migrating posteriorly. As it passes the remaining nephrogenic IM, the nephric duct sequentially induces formation of pronephric and mesonephric tubules. Upon reaching the posterior region, it sprouts a branch called the ureteric bud (UB) that invades the metanephric blastema. This process initiates the reciprocal tissue interactions that govern subsequent development of the metanephric kidney. These early events, from gastrulation through early branching morphogenesis, form the basis on which methods for de novo generation of renal tissues have been built.

Gastrulation comprises a complex series of cell movements and fate decisions that segregate the embryo into three germ layers: the ectoderm, mesoderm, and endoderm. Although gastrulae of different species exhibit marked morphologic variation, numerous studies have demonstrated that the molecular circuitry underlying this process is highly conserved. A combination of TGFβ/nodal/activin and WNT/β-catenin signaling are essential for specification of the endodermal and mesodermal lineages, while the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) pathways also contribute important, albeit less well defined, functions during gastrulation. Together these signals act in concert to direct the organized migration of cells toward their eventual fate and position in the embryo. In amniote embryos, such as birds and mammals, this results in the formation of a visible structure on the posterior half of the embryo—the primitive streak—through which mesendodermal progenitors ingress and differentiate. Recapitulation of the induction of gastrulation, or a primitive streak-like stage, is the foundation of many methods that attempt to differentiate pluripotent cells into renal lineages.

How are the different mesodermal lineages segregated during gastrulation? Initial insights were gathered using lineage-tracing experiments in chick and mouse embryos, which demonstrated that a cell's location within the primitive streak predicts its subsequent fate [7–10]. Specifically, the anteroposterior axis in the streak translates into the mediolateral axis of the embryo: the medially located paraxial mesoderm is derived from anterior streak while the posterior portions form the eventual LPM. The IM comes from an intermediate position, found to be roughly one-third of the streak length posterior to the node [8] (Fig. 44.1A). These findings, as well as similar pregastrula fate mapping data in zebrafish and *Xenopus* embryos [11–13], raise the possibility that cells are committed to the IM fate at a very early stage as they emerge from the primitive streak. However, contrary to

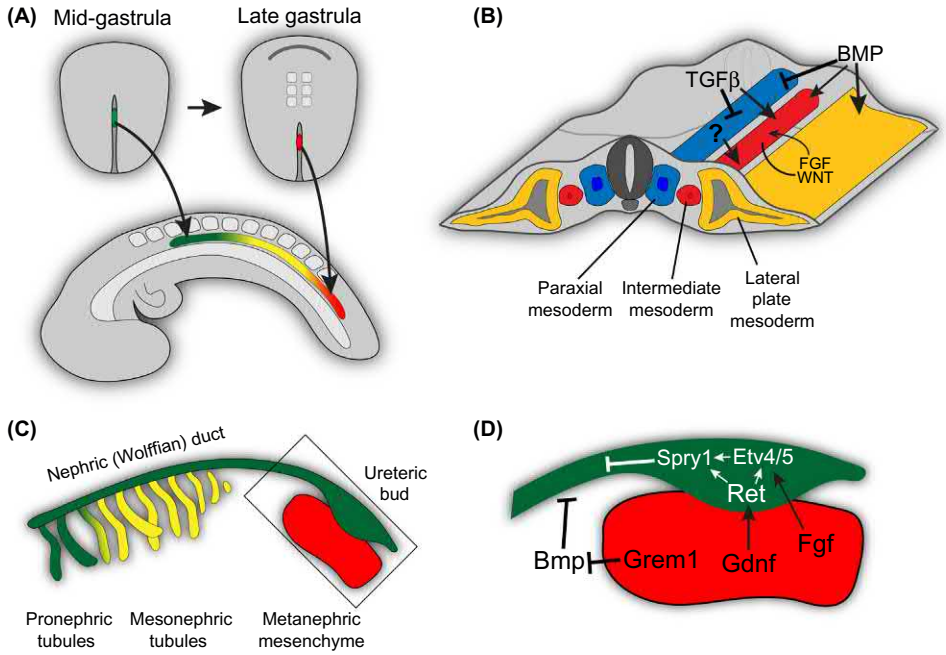

FIGURE 44.1 Early stages of vertebrate kidney development. (A) Presumptive IM cells transit through the mid-primitive streak, with earlier cells contributing to anterior tissues and later cells adopting a posterior fate. (B) Schematic representation of signaling pathways that mediate medial-lateral patterning of early mesodermal tissues. (C) The nephric duct derives from anterior pronephric IM and migrates posteriorly to contact the metanephric mesenchyme. There it develops a thickened UB, which ultimately migrates into the metanephros. (D) Multiple signaling pathways, including Gdnf, Fgf, and BMP, function to coordinate proper location of UB outgrowth. *IM*, Intermediate mesoderm; *UB*, ureteric bud.

this notion, there has been a myriad of experiments across several model organisms demonstrating marked plasticity among the early mesodermal lineages. Thus available data indicate that a cell's fate is instead determined by local signaling cues along the mediolateral axis (summarized in Fig. 44.1B). The BMP pathway has been shown to promote specification of lateral lineages while suppressing the paraxial mesodermal fate [14–16], and *Bmp4* is expressed in both the surface ectoderm and LPM itself at the appropriate developmental time [17]. Explant studies with chick mesoderm suggested a morphogen model in which high BMP promotes LPM, and intermediate signaling activity specifies the IM fate [18]. However, this model is likely oversimplified given that other signals are also involved. TGFβ is required for IM specification in chick mesoderm [19,20], consistent with previous observations that activin can promote pronephric gene expression from *Xenopus* animal caps [21]. Other important pathways include FGF and WNT, as FGF widens the IM field [22], and canonical WNT activity is required for the normal expansion of the IM progenitors [23]. Further, there is an unidentified medial signal, derived from the paraxial mesoderm, that is necessary for IM specification [24,25].

Collectively, the above data suggest that there is a complex milieu of growth factors that act in concert to pattern mesodermal lineages according to their mediolateral positions. Then once the initial blueprint is established, these tissue boundaries are further refined and sharpened by gene regulatory networks controlled by compartment-specific transcriptional regulators [26–28], a commonly observed mechanism throughout development [29]. Despite the tremendous advances in our understanding of these early events, there remains a largely unexplained phenomenon that the IM itself only forms in the caudal half of the embryo. This could be rationalized by one of two possible scenarios: (1) that the particular IM-inducing environment is not found in the rostral mesoderm, or (2) that anterior tissues are somehow refractory to adopting the IM fate. An elegant set of recombination experiments in chick embryos by Barak et al. provided support for the latter possibility [30]. Prospective IM tissue from the primitive streak transplanted into anterior regions adopted renal-specific gene expression (Lim1 and Pax2), while prospective nonnephrogenic tissue from an earlier stage streak was unresponsive to signals in the posterior IM. Hence, these results indicate that appropriate inductive cues are present along the anteroposterior axis in the prerenal mesoderm, but a distinct signaling environment in the primitive streak is required for cells to be competent to adopt the IM fate. One factor that could mediate this effect is retinoic acid (RA), which is both necessary for IM specification [31] and sufficient to cause ectopic anterior expansion of IM gene expression [20]. In chick the RA effect is phenocopied via misexpression of HoxB4, implying that RA is an important regulator of anterior–posterior axis formation.

While we have thus far focused on the specification of the IM as a whole, it is essential to also appreciate the domains along the longitudinal axis within the IM itself. These distinct anteroposterior identities are functionally realized at later stages of development. The anterior region, the site of formation of the pronephros, is unique in its ability to form the nephric duct that extends caudally to induce a wave of induction and tubulogenesis within the nephrogenic cord [32]. The posterior-most IM, where the metanephros forms, develops into the highly branched kidney exclusive to amniotes, while the intervening mesonephric IM forms a simpler pattern of parallel tubules along the trunk of the embryo. The molecular and developmental bases for this patterning are not well understood. Regionalized expression of important regulatory genes is evident soon after IM formation, such as *Gata3* in the rostral IM [33] and *Eya1* in the caudal domain [34]. Other genes, including *Sall1* and *Six1/4*, are broadly expressed but have functions that are specific to the prospective metanephric region [35,36]. To a large extent, though, the foundation of the anteroposterior arrangement within the IM is likely attributable to *Hox* factors, which broadly mediate axial patterning in mesodermal lineages (reviewed in Ref. [37]). Similar to other tissues, *Hox* genes are expressed in the early IM in a collinear order according to their chromosomal positions [20,38]. The posterior *Hox* cluster, particularly the *Hox10/11* paralogs, are crucial for proper development of the metanephric kidney [39–42]. Remarkably, ectopic expression of a *Hoxd11* transgene is sufficient to induce partial metanephric character in the mesonephros [43], consistent with the pattern of posterior dominance described elsewhere [44].

The mechanisms that control axial patterning are poorly understood, likely owing to the dynamic nature of gastrulation and iterative use of signaling pathways that complicate experimentation. It is known that the *Hox* code is established quite early in the embryo [45], even prior to expression of IM-specific genes. Accordingly, Attia et al. used nephric duct-forming capacity as a property specific to pronephric IM to demonstrate in avian embryos that anteroposterior pattern is determined during the primitive streak stage [46]. When prospective pronephros tissue was transplanted from the mid-streak to a comparable position in a later-stage embryo, it migrated and contributed to the posterior IM but still readily formed ectopic nephric duct structures. In the converse experiment, tissue transferred from the later to earlier streak still adopted the IM fate, but its cells did not contribute to the nephric duct. These intriguing results support a model in which the timing of a cell's ingression

through the streak determines its subsequent position along the longitudinal mesodermal axis, with later time points adopting more posterior fates, and its anteroposterior character is fixed at that time. From such a model, one might infer that the signaling environment within the streak is temporally evolving such that it has a more posteriorizing property at later stages, but to our knowledge this has not been demonstrated mechanistically. Candidate pathways that might underlie such a process include TGFβ and FGF, which have anteriorizing and posteriorizing effects, respectively, on the patterning of the nearby paraxial mesoderm [47,48].

Development of the nephric duct and ureteric bud

Following specification of the IM, the pronephric region develops a thin evagination at its dorsolateral boundary that buds to form the nephric duct. This epithelial tube then extends caudally along the length of the embryo, eventually forming the urinary collecting system as well as some components of the male reproductive system. The importance of the nephric duct and its derivatives has been extensively documented; for instance, mechanical impedance to its migration is sufficient to abolish all subsequent renal tubular differentiation in the neighboring IM [32]. The mechanisms underlying induction of the nephric duct, aside from those relevant to the formation of the pronephric IM itself, are poorly understood. Several transcription factors, including Pax2, Pax8, Gata3, Lhx1, and Hnf1β [33,49–51], are essential core components of a gene regulatory network that maintains the nephric duct identity [52]. However, the extrinsic cues that promote segregation of the duct from the remaining nephrogenic IM remain largely unknown.

The nephric duct migrates posteriorly in a stereotypic fashion until it reaches and fuses with the primitive cloaca, suggesting that it is driven by a highly orchestrated set of signaling cues and tissue interactions (reviewed in Ref. [53]). However, comparatively little is known regarding the mechanisms involved in this process. It has recently been shown that the remarkably straight migratory course of the nephric duct is likely guided by a strong attraction to the nearby column of IM [54], although the molecular basis for this observation is not understood. The key signals that drive caudal elongation include FGF ligands, such as Fgf8, which are derived from a posteriorly moving wave of expression that coordinates duct growth with body axis extension [54,55]. Intracellularly, the MEK/ERK pathway is essential for the leading nephric duct cells' response to FGFs, although other downstream signaling components may also play a role. Evidence from axolotl embryos suggests that glial cell-derived neurotrophic factor (GDNF) is another important chemoattractant for the migrating duct [56], but GDNF has been much more extensively studied for its role in morphogenesis of the UB (discussed later). As it grows caudally, the nephric duct also matures structurally from a loose cord of cells into a polarized, simple cuboidal epithelium. It reaches and inserts into the cloaca by embryonic day (E) 9.5 in the mouse embryo, governed by a complex regulatory cascade, at what will ultimately become the vesicoureteral junction [57–60].

Near its distal terminus, in the region of the metanephros, the nephric duct develops an epithelial outgrowth termed the UB. This structure will ultimately form the upper urinary tract, including the ureter, renal pelvis, and intrarenal collecting duct system. Moreover, it plays a fundamental and indispensable role in kidney development. Given that its function is restricted to the metanephric kidney, the UB has principally been studied in the context of mouse embryology, and a wide breadth of genetic studies has uncovered an intricate set of underlying signaling mechanisms (reviewed in Ref. [61]). The first morphologic suggestion of the UB is a club-like thickening of the nephric duct epithelium adjacent to the condensed metanephric mesenchyme (MM) at E10.5 (Fig. 44.1C and D). Within 24 hours, the tall pseudostratified epithelium elongates to form a branch that migrates and invades into the adjacent metanephric blastema. Appropriate positioning of UB outgrowth is crucial; thus it is precisely controlled by a number of positive and negative regulators. Loss of inhibitory signals results in ectopic or malpositioned UBs, which ultimately causes impaired function of the urinary tract. Conversely, failure of the requisite stimulatory molecules to induce proper UB growth leads to severely perturbed kidney development including renal agenesis.

The molecular linchpin of UB induction is GDNF, which is expressed by the MM and signals through the receptor tyrosine kinase (RTK) RET on the adjacent nephric duct cells. Loss-of-function mutations in either *Gdnf* or *Ret*, and even the coreceptor *Gfr1a*, result in failure of UB outgrowth [62–66]. Further, an ectopic source of GDNF is sufficient to induce numerous UBs along the nephric duct [67], indicating that strict regulation of GDNF localization is essential for normal kidney development. Accordingly, *Gdnf* transcription is tightly dependent upon a complex combination of factors that is unique to the MM, including Eya1, Hoxa11/Hoxd11, Six1, Sall1, and Pax2 [68]. In addition, *Gdnf* expression is actively restricted from more anterior regions of the nephrogenic cord by Slit/Robo signaling and FoxC transcription factors [69,70]. Further layers of control over UB outgrowth exist within the nephric duct itself and its cells' ability to respond to induction signals. First, it was shown through chimeric embryo experiments that

RET-dependent competitive cell movements result in convergence of the most signal-receptive cells to a single location in the duct, and this population likely inhibits the neighboring cells from forming additional buds [71]. Second, there are negative feedback regulators, such as Spry1, that antagonize intracellular responses to RTK signaling to locally confine the effect of secreted GDNF [72,73].

Aside from GDNF/RET-driven processes, there are other pathways that further reinforce proper timing and location of UB induction. FGF signaling, including Fgf10 in particular, was found to promote UB development, albeit to a lesser extent than GDNF. In rare cases, *Gdnf* or *Ret* mutant embryos do form primitive buds; strikingly, simultaneous deletion of the antagonist *Spry1* restores fairly normal UB growth in these mutants. In this setting, Fgf10 is indispensable for rescue of UB initiation [74], and it similarly functions through an RTK to activate essential downstream transcription factors *Etv4/5* and *Sox8/9* [75–77]. Conversely, BMP signaling functions to repress UB outgrowth in a pathway parallel to the GDNF/RET axis. Reduction of *Bmp* gene dosage can result in ectopic budding, while addition of exogenous BMP blocks UB growth in ex vivo organ culture [78,79]. Under physiologic conditions, the secreted BMP antagonist Gremlin1 is specifically localized in the MM to mediate derepression of UB formation [80–82].

The UB initially grows as a straight duct into the MM, but by E11.5 the ureteric epithelium initiates an extensive, stereotypic branching program that ultimately produces an elaborate tubular tree. The predominant mechanism driving this process is iterative bifurcation (and less commonly trifurcation) at the tips of the network, which produces >2000 branching events by E16.5 [83]. During branching, the UB is organized into molecularly distinct domains (Fig. 44.2A) with progenitor cells occupying the tip compartments while cells of the "stalk," or "trunk," adopt a more differentiated collecting duct phenotype [84]. The ureteric tip cells are thus essential to normal kidney organogenesis, but isolated ureteric stalks also demonstrate a capacity for regenerating the tip domain and branching program [85]. The maintenance, proliferation, and morphogenesis of the ureteric progenitors are directed by a complex signaling milieu that includes several of the pathways involved in initial UB outgrowth. Again GDNF/Ret and FGF are the dominant pathways that promote proliferation and budding [73,86–90], while BMP signaling antagonizes branching morphogenesis in the stalk domain [91]. The canonical WNT pathway has both cell- and noncell-autonomous roles in ureteric branching; its transcriptional effector Ctnnb1 is required for maintenance of tip identity and morphogenesis [92,93]. Wnt11, whose transcription is specific to tip cells and is RET-dependent, signals to the adjacent mesenchyme to augment *Gdnf* expression as part of a positive feedback mechanism [94]. Disruption of this loop via *Wnt11* knockout results in hypoplastic kidneys. Stromal-derived RA is also required for UB development, in part through direct maintenance of *Ret* expression [95,96]. Finally, Hedgehog pathway activity is actively suppressed in the branching portion of the ureteric tree to prevent precocious loss of progenitor cells [97]. Further work will be necessary to dissect the epistatic relationships among these signaling pathways and to elucidate how they cooperate at the cellular level to coordinate ureteric development.

Maintenance and differentiation of the nephron progenitor cell

By the time of UB outgrowth, the caudal portion of the nephrogenic IM has condensed into a histologically distinct blastema, called the MM, which is a multipotent population of cells. As the UB begins to branch, a large portion of the MM cells organize around the UB tips into domains of cap mesenchyme (CM). The presence of CM persists at the UB tips throughout branching morphogenesis, and it serves several critical functions during kidney development. First, it provides essential signals, such as GDNF, to maintain proliferation and budding of the developing ureteric epithelium. Second, through elegant lineage-tracing experiments using the CM markers *Six2* and *Cited1*, it was demonstrated that CM cells are a self-renewing population of multipotent progenitors that ultimately differentiate into all of the epithelial cells of the nephron from the glomerulus to the connecting tubule [98,99]. Thus in light of this potential, cells of the CM are also referred to as nephron progenitor cells (NPCs). The NPCs and UB tip progenitors are codependent and form intricate reciprocal signaling loops that preserve the nephrogenic niche (Fig. 44.2A). Stromal progenitor cells also contribute to the niche, and they will be discussed in the subsequent section. Here we will focus on the mechanisms that promote maintenance, induction, and differentiation of the NPCs.

Like most stem or progenitor cell populations, balance between NPC self-renewal versus differentiation is regulated by a network of signals within the nephrogenic niche. Perturbation of this equilibrium typically results in hypoplastic and/or dysplastic kidneys. A key transcriptional regulator of the multipotent state is Six2, which is required to prevent precocious differentiation and tubulogenesis in the NPCs [100]. Further, it has more recently been shown that downregulation of Six2 is required for differentiation, as transgenic embryos that maintain Six2 fail to form nephron segments [101]. As is the case with UB progenitors, RTK signaling promotes the survival and

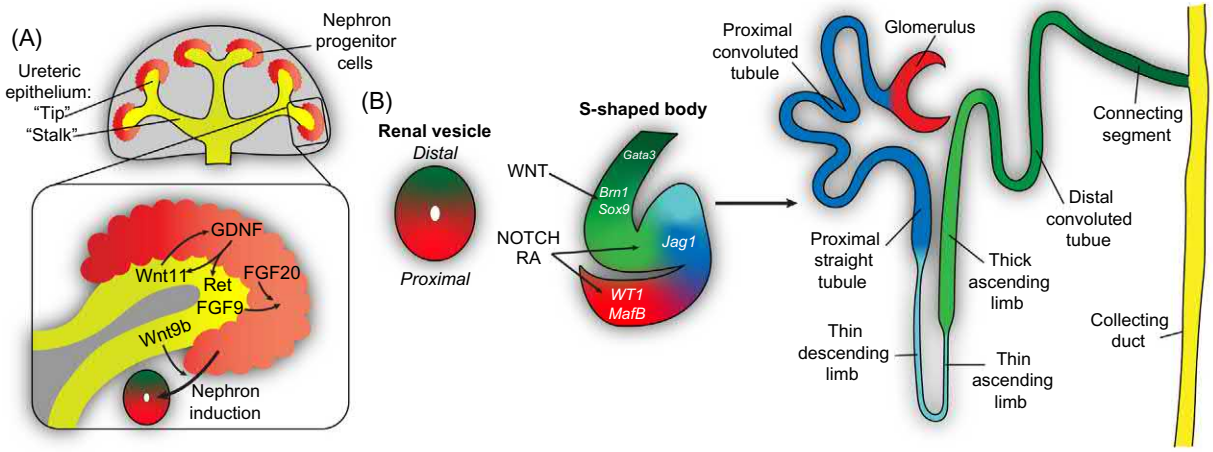

FIGURE 44.2 Overview of metanephric nephrogenesis. (A) Reciprocal interactions between the cap mesenchyme and ureteric epithelium regulate branching events and maintenance of nephron progenitor cells. (B) Following induction, nephron progenitors progress through renal vesicle, comma-shaped body, and S-shaped body stages of differentiation. The S-shaped body is highly organized at the molecular level into distinct domains that will give rise to each of the segments of the mature nephron.

proliferation of NPCs, primarily through the FGF pathway [102,103]. The essential ligands are FGF9 and FGF20, which derive from the UB and CM, respectively [104]. The existence of redundant paracrine and autocrine signals reinforces the importance of NPC maintenance in kidney development, although the genetic circuitry underlying these expression patterns is not well understood. *Bmp7* is broadly expressed in the early kidney, including in both the ureteric epithelium and CM, and it contributes to the renewal of the NPC compartment [105–107]. Interestingly, Bmp7 functions in a Smad-independent manner, instead signaling through MAPK and JNK pathways to maintain the NPCs [108–110]. In somewhat paradoxical fashion, Bmp-mediated SMAD activation actually sensitizes NPCs for induction and causes them to move toward the more differentiated compartment [111,112]. The other major pathway responsible for NPC proliferation is Wnt/β-catenin signaling, which has also been studied for its role in induction/differentiation. Embryos deficient in *Wnt9b*, secreted from the ureteric epithelium, exhibit severe renal hypoplasia [113]. Consistent with a UB-to-CM signaling paradigm, deletion of *Ctnnb1* in the MM phenocopies the *Wnt9b* mutant and results in loss of NPC markers [114,115]. Conversely, genetic activation of the canonical Wnt pathway induces expansion of Six2-expressing NPCs, although nephrogenesis appears somewhat dysplastic [93]. At the molecular level, data from ChIP experiments showed that Ctnnb1 and Six2 can bind an overlapping set of enhancers, suggesting that they may cooperate to regulate a progenitor cell gene regulatory network [116].

At each new branch tip in the developing ureteric tree, a subset of NPCs are induced to condense into a structure called the pretubular aggregate (PTA). Cells in the PTA initiate expression of genes associated with their induced state (*Lhx1*, *Pax8*, *Wnt4*, and *Lef1*) and downregulate NPC genes as they differentiate, although a low level of Six2 expression is still detectable at this stage [117]. PTAs subsequently undergo a mesenchymal-to-epithelial transition (MET) event to form a polarized renal vesicle (RV). Further stereotypic morphogenetic processes transform the RV into an S-shaped body (SSB), named for its appearance in two-dimensional cross section, which already contains the molecular information that will establish the proximal–distal axis of the future nephron (Fig. 44.2B). Recent studies used a combination of time-lapse imaging and genetic lineage labeling to demonstrate that NPCs migrate through the niche and encounter signaling cues in a stochastic manner, and there is also a newly appreciated plasticity at the NPC-PTA differentiation boundary [118,119]. Our current understanding of the inductive events that lead to NPC differentiation is not complete, and it features many of the same pathways implicated in self-renewal. Wnt ligands play a central role, as UB-derived Wnt9b is essential for induction and tubulogenesis [113], as well as its function in the maintenance of progenitor cells. In addition, *Wnt4* is expressed from the PTA itself and is required for MET and differentiation [120–122]. From a mechanistic perspective, *Wnt4* expression is lost in *Wnt9b* mutants, suggestive of an inductive cascade whereby Wnt9b induces initial nephron commitment and transcription of *Wnt4*, which then promotes further differentiation. Interestingly, Wnt4 functions primarily through a noncanonical Wnt/Ca pathway [123,124], consistent with the observation that prolonged stabilization of Ctnnb1 inhibits epithelialization [115].

The finding that Wnt9b can promote both NPC proliferation and differentiation is somewhat perplexing; recent evidence has led to a model in which low-level activation of Ctnnb1 promotes proliferation and high activity drives differentiation [125]. The PTA also expresses *Fgf8*, which is required for the survival and proliferation of the nascent epithelium [126], and this signal appears to function distinctly from the Fgf9/20 that promotes NPC maintenance. How do Wnt and FGF, as well as BMP as described previously, stimulate both self-renewal and induction of progenitors? One possibility is signaling gradients and graded responses, such as that postulated for the Wnt/ß-catenin pathway. An alternative hypothesis is the activity of yet another pathway that can alter the response of a cell to a given signal. Indeed, it has recently been demonstrated that Notch activity is both necessary and sufficient for NPCs to downregulate Six2 and differentiate into epithelial nephron derivatives [101,127,128]. In fact, Notch activation is sufficient to induce MET and tubulogenesis even in the absence of both Wnt9b and Wnt4 [129]. In the native setting, it is unknown which cells are providing Notch ligand to promote differentiation.

Key to the postnatal kidney's remarkable ability to maintain fluid and electrolyte homeostasis is its highly ordered functional segmentation along the proximal−distal axis. This pattern is initially established early during nephron induction, largely through activation of a complex set of transcription factors that will ultimately precisely control the expression of segment-specific channels and transporters [130]. The SSB is organized into morphologically and molecularly discrete proximal, medial, and distal domains (Fig. 44.2 and reviewed in Ref. [131]). Further, single cell transcriptional profiling demonstrated that there is already polarized gene expression within the RV itself, with the proximal portion enriched for genes associated with podocyte fate and distal tubule markers found more selectively in the distal RV [132]. This pattern is not fully refined, however, as a large number of cells express genes associated with multiple lineages. Scrupulous analyses of both mouse and human fetal kidneys have shed further light on this process, revealing that induced nephron progenitors sequentially exit the niche and successively build the presumptive nephron in a distal-to-proximal manner [133]. This suggests that there is a dynamic spatiotemporal signaling microenvironment at the UB tip that coordinates segmentation from the outset of nephrogenesis.

Given the dynamic nature of early tubulogenesis and the iterative use of the same pathways at multiple stages of development, it is challenging to isolate and identify the signaling mechanisms that control nephron segmentation. Despite their dramatically simplified anatomy, the pronephric nephrons of *Xenopus* and Zebrafish larvae exhibit subdivisions analogous to those observed in the metanephric tubule, and these organisms have thus been powerful models to identify the molecular basis of nephron pattern formation. Key signals for determination of proximal fates include Notch and RA. Notch activity promotes formation of proximal structures including podocytes and proximal tubules [134−136], and it functions primarily through the *Notch2* receptor in mammals [137,138]. RA similarly induces proximal and represses distal fates in the zebrafish pronephros [139,140], likely in part through activation of master regulators of tubular differentiation such as *Hnf1β* [141−143]. Assessment of epistatic relationships indicates that RA functions upstream of Notch during segmentation [144], but RA's role in this process has not been tested in mammals. In opposition to these signals is the distalizing Wnt/β-catenin pathway, which forms a morphogen patterning gradient during conversion to the SSB stage [145]. This gradient is also modulated by BMP/PI3K activity that is required for specification of the medial segment and its derivates. Further work is necessary to identify mechanisms of cross talk among these various pathways and how they are integrated to effect cell fate decisions.

Role of stromal lineages in kidney organogenesis

Aside from the NPC-derived epithelial nephron elements and the UB-derived collecting duct system, the other major lineage constituting the kidney is the stroma. This compartment is extensive and includes the glomerular mesangial cells, cortical and medullary interstitial cells, and the highly specialized renal vasculature. Embryologically, these cells largely develop from a population of Foxd1-positive stromal progenitor (SP) cells that surround the CM at the periphery of the kidney. The SP cells are a self-renewing population that, in a manner analogous to NPCs, generate differentiated cell types in a radial, inside-to-outside pattern [146]. The initial origin of the stromal lineage is not entirely resolved. Similar to both the UB and NPCs, the SP cells descend from early Osr1-expressing precursors that are no longer detectable by E11.5 [147]. However, because Osr1 is broadly expressed in both the early IM and LPM, this does not necessarily reflect a shared origin. In fact, clonal analysis via cre/loxp-mediated lineage labeling failed to identify single cells that give rise to both the NPC and SP lineages, suggesting that they arise independently despite their close spatial relationship. On the other hand, several groups have identified occasional instances in which Foxd1 and Six2 are coexpressed, and Foxd1-positive cells sometimes generate renal tubules [132,146]. Further, it was recently found that sustained expression of Pax2 is required in NPCs to actively repress stroma-associated transcriptional programs [148]. These latter experiments lend strong support to a shared lineage model, which may

have previously gone undetected due to technical limitations associated with lineage-tracing experiments. Regardless, new analyses of fetal kidney tissue unexpectedly revealed that there is persistent and considerable overlap between the Six2 and Foxd1 compartments in humans, appreciable at both the transcriptional and protein levels. Thus lineage boundaries are more imprecise or ambiguous in man [149].

While the reciprocal tissue−tissue interactions between the NPCs and UB have been well documented over several decades, we are now gaining more appreciation for the SP cells' contribution to the nephrogenic niche of developing kidneys. Given their juxtaposition to the NPCs in the developing cortex, they occupy a position well suited to impact nephron development. Genetic ablation of the SP lineage results in a striking phenotype in which NPCs accumulate at the ureteric tips and are unable to differentiate [150], suggesting that the stroma may provide permissive signals required for NPC induction. Similar but less extensive phenotypes have been observed in the setting of *Foxd1* deletion, which also results in abnormal ureteric branching and development [57,151]. Candidate prodifferentiation mechanisms downstream of Foxd1 include signaling molecules that impact Wnt, BMP, and Hippo activity within NPCs [150,152], although it is unclear whether the SP cells signal directly to the NPCs or do so indirectly via another cell type. The stroma is also the source of RA, which is required to maintain normal ureteric branching as previously discussed [95].

While the Foxd1 lineage unambiguously gives rise to mesangial and interstitial cells, the origin of the renal vasculature is less clear. The predominance of lineage-tracing data indicate that *Foxd1*-positive cells do not contribute significantly to endothelial cells [146,153], although one group has found it to have substantial contribution to the endothelium [154]. The explanation for the discrepancies between these findings is unclear but could be due to technical differences (i.e., mouse strain, knock-in vs transgenic). Rigorous morphometric analyses revealed that early endothelial progenitors both surround the nascent metanephric blastema and migrate into the developing kidney with a mesenchymal layer surrounding the UB [155,156]. These precursors elaborate organized plexuses around the growing ureteric tips and CM, and they subsequently develop in apparent coordination with early differentiating nephrons. A subset of endothelial cells invades a cleft in the proximal SSB that abuts the presumptive podocyte population. These cells will form the glomerular capillaries, and they also recruit differentiating SP cells to the mesangial compartment in a PDGF-dependent manner [157]. Mesangial cells differentiate under the guidance of Notch signaling, and they are required in turn for further maturation of the glomerular capillary tuft [153].

Nephron endowment

The iterative cycle of UB branching and NPC induction is a powerful mechanism that generates exponential kidney growth and nephron formation [83]. The period of nephrogenesis is finite, however, as the progenitor cells in the niche ultimately are concomitantly depleted in a terminal wave of differentiation [158]. This occurs in the early postnatal period in rodents [159] and sometime around the 36th week of gestation in man [160]. Consequently, individuals are endowed with a fixed number of nephrons that is subject to gradual decline over a lifetime. The supply is highly variable in humans, with up to a 10-fold range reported, and those allotted fewer are at higher risk for complications later in life including hypertension and chronic kidney disease (CKD) [161]. Here we will provide a brief discussion of the genetic and environmental determinants that regulate the timing and extent of nephrogenesis, which overall are not well understood.

The close temporal association between birth and the cessation of nephrogenesis in mice bolsters a model in which some parturition-related signal leads to the en masse differentiation of remaining CM cells. However, because this event normally occurs in utero in humans, there is more likely an intrinsic mechanism that drives terminal differentiation. Although perturbation of any component within the niche could disrupt self-renewal, the NPCs have been the subject of more intense focus given that they are the first observed to lose their progenitor gene expression [158]. One hypothesis is that gradual reduction of NPCs over time alters the signaling environment to ultimately tip the balance in favor of differentiation. Yet when NPC depletion is accelerated through genetic ablation, which results in hypoplastic kidneys with fewer nephrons, nephrogenesis still persists through the same timepoint [162]. Thus the temporal control seems to be more actively regulated. Through a series of heterochronic transplantation experiments in kidney explant cultures, Chen et al. observed that older NPCs exhibit impaired ability to integrate within the nephrogenic niche [163]. These data imply that the progenitor lifespan may be constrained, and NPCs are therefore more analogous to a transient amplifying population than true stem cells. The basis for this progenitor aging is entirely unknown, and it is not readily apparent in transcriptomic comparisons.

While many have described genetic manipulations that disrupt the signaling circuitry in the nephrogenic zone and cause precocious loss and differentiation of NPCs, phenotypes with extended nephrogenesis are very rare. Recently, it was found that forced expression of *Lin28b*, a regulator of miRNA biogenesis with known roles in several stem cell types [164], is sufficient to prolong

nephrogenesis for an additional several weeks [165]. Surprisingly, this phenotype was observed when transgene expression was driven in the *WT1* but not the *Six2* lineage [166]. Further, embryonic kidneys were refractory to the effects of *Lin28b* when induced after E16.5, suggesting that there is an unknown mechanism that impacts plasticity in a temporal-dependent manner. There is also emerging evidence that metabolic pathways might regulate the timing of kidney development. Increased mTOR activity via reduction of *Tsc1* leads to slightly prolonged nephrogenesis and enlarged kidneys [167]. Accordingly, NPCs appear to shift from glycolytic to aerobic metabolism as they transition from self-renewal to differentiation [168], and calorie restriction during pregnancy leads to hypoplastic kidneys [169]. Overall there remains much to uncover regarding the mechanisms driving cessation of nephrogenesis, but it seems likely to involve an interface between environmental factors and the developmental signaling pathways that are known to control renewal and differentiation.

Kidney repair and regeneration

In the clinical setting, the kidney is subject to a number of insults over a lifetime, including ischemic injury, nephrotoxins, and stress associated with chronic and systemic diseases. In response to acute tubular injury, tubular epithelial cells exhibit a significant capacity for repair. However, the recovery process is limited to the regeneration of surviving tubules as it is widely accepted that no new nephrons form beyond the fetal/perinatal period. Further, repair is typically incomplete, and thus renal reserve and kidney function ultimately decline over time with severe or repetitive injury. In the setting of chronic stress, maladaptive mechanisms predominate and lead to fibrosis and CKD [170]. Cumulatively, this leads to a staggering and ever-increasing number of patients that require renal replacement therapy in the form of either dialysis or kidney transplantation. As a result, there are intensive efforts ongoing on several fronts to improve patient outcomes and ease the enormous economic burden associated with CKD. Further elucidation of the normal renal repair mechanisms is essential to help guide development of therapeutic strategies to mitigate injury and augment recovery. Given the significant scarcity of organs available for transplantation relative to the current demand, there is growing interest and enthusiasm for cell- or tissue-based therapies that may over time be able to replace native renal function in individuals with kidney failure. Along these lines, tremendous progress has been made over the past several years in developing methods to generate renal tissues, particularly in the field of stem cell differentiation.

Stem cells in kidney repair

Under normal conditions, the kidney does not exhibit significant turnover or self-renewal like that seen in the skin, GI tract, and blood, and it is therefore thought to be a relatively static organ with respect to its cell populations. Given its ability to initiate proliferation and regenerate tubules subsequent to injury, it has long been speculated that the kidney might contain a small population of quiescent resident stem cells that are activated during the repair process. However, although there are some rare cells in the adult kidney with features similar to tissue stem cells found in other organs [171–173], the predominance of available evidence argues against the existence of a functional renal stem cell. Humphreys et al. in our laboratory first used *Six2*-Cre lineage tracing to demonstrate that regenerated tubules derive from the epithelialized compartment rather than an interstitial cell type [174]. While these experiments do not rule out the possibility of a *Six2*$^{+}$-derived stem cell, it has since been demonstrated through similar techniques that differentiated proximal tubule cells (expressing *Slc34a1*) are the source for the bulk of the regenerated proximal tubular mass [175]. These data therefore support a model in which de-differentiated epithelial cells, instead of stem cells, proliferate and expand in response to injury to restore kidney function. This was further strengthened by single cell labeling studies that showed that regenerative clones are restricted to a single segment fate along the length of the nephron [176,177]. Thus not only do regenerated tubules derive from differentiated epithelia, but there is also no evidence of multipotency or plasticity between cell lineages, which is a defining characteristic of stem cells. With respect to glomerular lineages, several groups have reported populations that exhibit transdifferentiation of *Renin*-expressing cells of the juxtaglomerular apparatus [178–180] and parietal epithelial cells of Bowman's capsule [181,182] into podocytes in the setting of glomerular injury. However, the overall contribution and significance of these lineages in repair and regenerative processes is not fully characterized.

While the aforementioned studies establish differentiated epithelia as the source of regeneration, they do not distinguish whether this property is shared among all tubular cells or limited to a specialized subpopulation. To address this question, proliferating cells in the kidney were sequentially labeled with different thymidine analogs during the acute phase of injury repair. Under these experimental conditions, there was minimal overlap of incorporation of both markers, suggesting that tubular cells divide in a stochastic and limited manner [178]. These data make a compelling argument against the existence of a dedicated progenitor within the epithelium, which would have to undergo numerous rounds of cell

division and thus incorporate multiple labels. At baseline, a small subset of tubular cells expresses the developmental transcription factor *Sox9*, which marks progenitor cells in other organs. This gene is broadly upregulated during the proliferative response to injury, and it is essential for proper tubular regeneration [179]. While these observations raise the possibility that the preexisting Sox9-positive cells are progenitors, they do not preferentially contribute during the repair process, further corroborating the model of stochastic survival and proliferation of tubular cells. With the increasingly widespread use of single cell sequencing technologies, it is likely that heterogeneity within the renal parenchyma will become more and more evident, which will permit further genetic investigation of possible sublineage involvement during injury repair.

The molecular events of tubule regeneration following injury have been intensely studied [180]. One striking feature associated with proliferative epithelial cells is the reexpression of developmental regulatory genes including Pax2, Sall1, and Sox9 [179,181,182]. This phenomenon implies that a dedifferentiation step may be critical for the repair process [183]; however, tubular cells do not reexpress genes, such as Six2, that would indicate full reversion to a NPC-like state [174]. This is consistent with the clonal lineage restriction observed in regenerated tubules [176], which contrasts with the multipotent character of NPCs. In fact, the inability to generate nephrogenic cells with full developmental potential is likely a major underlying reason the kidneys cannot generate new nephrons outside of the fetal period. To determine whether there is ever a time at which the kidney would exhibit de novo nephrogenesis, our laboratory performed cryoablation injury studies in neonatal mice. Surprisingly, even as early as the first postnatal day, at which time NPCs are still proliferative and differentiating, injury does not result in compensatory expansion or prolongation of nephrogenesis [184]. In contrast, the kidneys of other species such as zebrafish contain multipotent nephron progenitors that can regenerate nephrons de novo in response to injury [185–187]. Thus it seems that the mammalian kidney is particularly refractory to neonephrogenesis, and any strategy to replace lost nephrons would have to rely upon mechanisms outside of its usual genetic or physiologic repertoire.

Sources of nephrogenic cells

To overcome the limitations of endogenous kidney regeneration, numerous strategies have been attempted in experimental settings to produce cells capable of de novo nephrogenesis. Drawing on experiences in several fields, including induced pluripotency, Hendry et al. identified a combination of embryonic transcription factors, including Osr1, Six1/2, Eya1, and Hoxa11, that is able to reprogram an adult kidney epithelial cell line into an NPC-like state [188]. Interestingly, the factor Snai2 is also required, highlighting the importance of epithelial-to-mesenchymal transition during this process of reversion to a fetal precursor. Although the resulting cells were able to integrate into the NPC compartment in ex vivo kidney culture, the method is inefficient and the progenitors are unable to differentiate given the continued forced expression of the reprogramming factors. This approach was recently refined to include only three factors (Six1, Eya1, and Snai2) and use of an inducible promoter that allows for silencing of the reprogramming cassette [189]. Under these conditions, induced NPC-like cells indeed incorporate into differentiated tubular structures of the neonatal mouse kidney. The area of direct reprogramming has not been further developed in the kidney, to our knowledge, but it may be a viable strategy in improve repair mechanisms. Fate conversion has been successfully performed in vivo in quiescent epithelial organs such as the pancreas [190], and viral delivery of developmental transcription factors was shown to ameliorate fibrosis and enhance epithelial recovery in liver disease [191].

Given that NPCs exhibit stem cell-like properties in vivo, albeit only over a limited time period, several groups recently explored whether these cells could be cultured and expanded in vitro. Exploiting developmental signaling principles, such as the FGF and WNT pathways, three sets of similar culture conditions were identified that can support serial passaging and propagation of progenitors [111,192,193]. These have been successfully applied to both mouse and human fetal NPCs, although the human cells first require enrichment through FACS based on cell-surface markers [193]. Brown et al. showed that their conditions can select and enrich for NPCs from a heterogenous population of cells from the nephrogenic zone of the fetal kidney, which could alleviate the need for FACS purification. Although the expression of several important factors, such as *Pax2* and *Wt1*, tends to wane over time, the cells retain high expression of other NPC genes and the capacity to differentiate in vitro into nephron elements for many passages. Interestingly, the cells propagated by Brown et al. did not form glomerular derivates when induced in aggregates. While this could reflect altered differentiation capacity, it is more likely due to culture conditions that persistently stimulate the WNT pathway, which is known to antagonize commitment to the podocyte fate [145]. To test in vivo potential, Zhongwei et al. engrafted NPCs into the mouse nephrogenic zone on postnatal day one, and the cells successfully integrated into developing host nephrons. Similar subcapsular injections into the adult kidney in a nephrotoxin AKI model did not yield functional engraftment during tubule regeneration, but it did somewhat improve AKI outcomes

by unknown mechanisms. Intraparenchymal delivery will likely be required to fully assess whether NPCs can assist in the repair process in the setting of acute or CKD.

For many tissues, especially those with well-characterized stem cell populations, the past decade has witnessed an eruption in the development and application of organoid technologies. Much of this work was pioneered in the GI tract by Hans Clevers' group in Utrecht. Typically, strategies for tissue-derived organoids rely on the recreation of the stem cell niche environment to support three-dimensional growth and differentiation of organ-specific progenitor cells, which are most often obtained from patient biopsy samples or sacrificed animals. Until very recently, this methodology had not expanded to include the kidney, likely owing to its lack of stem cells. Schutgens et al. have now described methods to produce kidney "tubuloids," which can be derived from core biopsy specimens or even cells isolated from patients' urine [194]. These three-dimensional structures can be established rapidly, and they expand with genetic stability demonstrated over numerous passages. From any given sample, a heterogenous mixture of tubuloids representing various nephron segments is generated, although the proximal tubule appears to be overrepresented. The epithelia retain the expression of differentiated markers associated with their origin, so it is unclear to what extent, if any, dedifferentiation of the tubular cells is required for their proliferation. Regardless, this system for rapid expansion of tubular cells, which can be done in a minimally invasive and patient-specific manner, represents another possible avenue for replacing cells lost or damaged in disease states. Further, there is more immediate potential for use in disease modeling, diagnostics, and drug testing.

Differentiation of renal tissue from pluripotent stem cells (organoids)

One prospective source of material for regenerative applications in the kidney is human pluripotent stem cells (hPSCs), including both embryonic stem cells (ESCs) and induced PSCs (iPSCs), which have the potential to differentiate into any cell type of the body. These cells could provide a limitless supply of materials for generating renal tissues, and in the case of iPSCs, they offer the ability to generate therapeutic materials that are isogenic to patients. While early work with hPSCs focused on simple, two-dimensional differentiation to generate single desired cell types, more recent advances have highlighted the ability to produce complex three-dimensional structures (usually termed organoids) that comprise numerous cell types and more accurately embody normal organ architecture [195]. Regardless of the culture format, prevailing strategies utilize a stepwise directed differentiation approach designed to recapitulate the natural developmental processes that occur during embryonic organogenesis. In this section, we will summarize how this has been applied to differentiation of hPSCs into the kidney lineage, a field that has seen astonishing growth and progress over just the past few years (reviewed in Ref. [196]).

Early attempts to differentiate renal tissue from hPSCs began with elucidation of mechanisms to specify the IM. In most cases, this appropriately proceeds first through induction of a primitive streak stage that produces an early mesodermal progenitor population, which is typically achieved via activation of a combination of WNT, BMP, and TGFβ pathways to mimic gastrulation [197–201]. Cells are then guided toward the IM fate by various combinations of signals including FGFs, RA, Activin, and BMPs. In some cases, hPSCs are differentiated directly to IM without an obvious mesodermal progenitor intermediate [202,203]. The wide array of methods that have been used to produce progenitors with overlapping molecular features implies that we do not yet fully understand the signaling mechanisms that direct IM specification. It also suggests that hPSC cultures themselves produce a significant complement of endogenous signals that likely support the various approaches to differentiation.

Building upon these previous studies, the laboratory of Melissa Little and our group concurrently developed efficient methods to differentiate hPSCs into MM and NPCs [204,205]. To provide a more physiologic environment, both groups introduced an aggregation step to force the early progenitors to adopt a three-dimensional configuration. A short pulse of induction signal to activate the canonical Wnt pathway, followed by several weeks of culture under permissive conditions, is then sufficient to induce widespread epithelialization and nephron differentiation. The resulting three-dimensional kidney organoids contain multiple nephron lineages, including podocytes, proximal tubule, loop of Henle, and distal tubule (summarized in Fig. 44.3). These structures represent a major advance in the field of kidney differentiation, and the protocols by which they are generated are now being widely applied to a variety of biological questions. Although they most closely resemble early fetal kidneys [206], the organoids have already proven to be tractable models for congenital kidney disease and tubular injury [205,207].

One of the more glaring limitations of the described hPSC-derived kidney organoids is that primarily only one of the major progenitor compartments, the NPCs, is well represented. Although Takasato et al. invoke the presence of putative collecting duct epithelium, several of its molecular markers are also found in other nephron segments [204,208]. Neither protocol leads to efficient production of early ureteric progenitors and organoids do not contain a branching tree that is the cornerstone of metanephric kidney development. This is not altogether

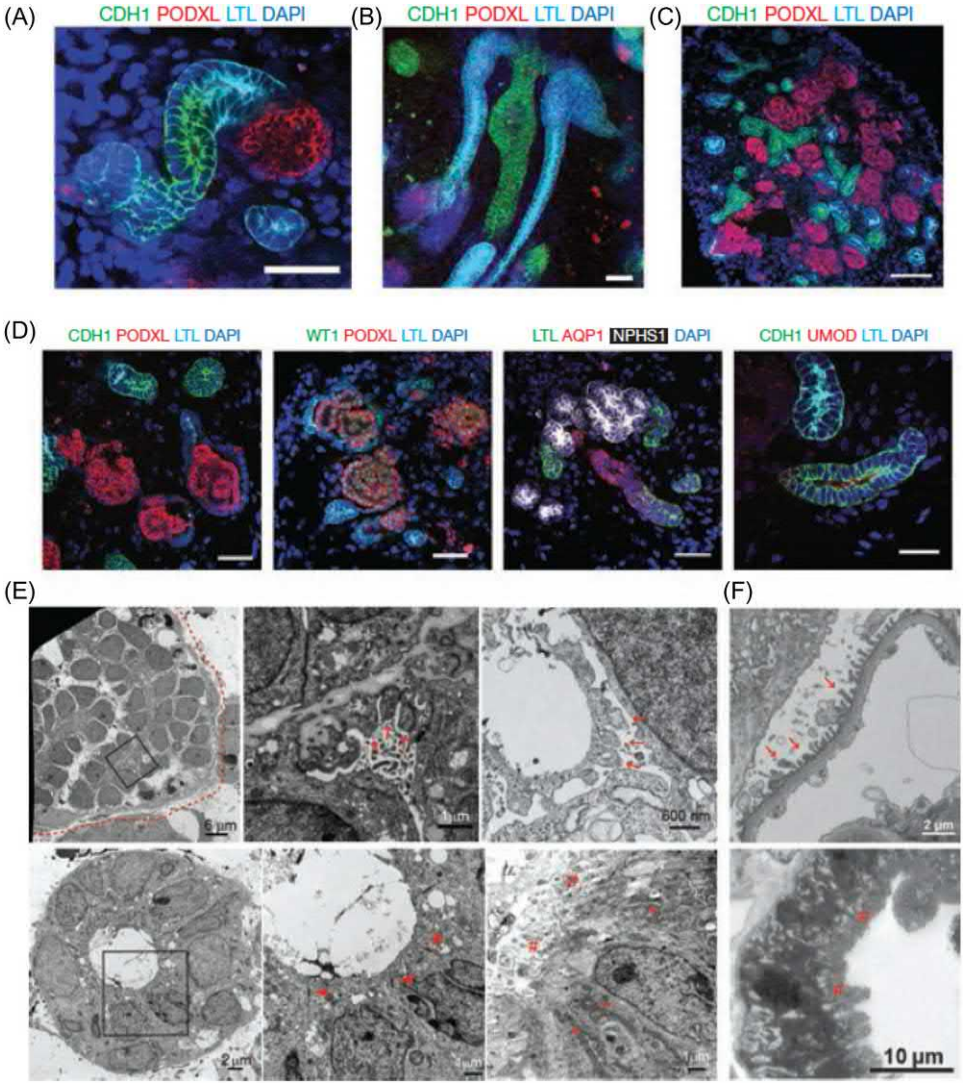

FIGURE 44.3 PSC-derived kidney organoids contain diverse nephron cell types and structures. (A–D) Immunofluorescent staining reveals the presence of glomerular (PODXL, WT1), proximal tubule (LTL, AQP1), and distal tubule (CDH1, UMOD) components within late stage human kidney organoids. Transmission electron micrographs of both human kidney organoids (E) and human kidneys (F) demonstrate similarities at the ultrastructural level, including podocyte foot processes (arrows), epithelial tight junctions (arrowheads), and tubular brush borders (pound sign). Scale bars, 50 µm, in (A–C). *Reproduced with permission from Morizane R, et al. Nephron organoids derived from human pluripotent stem cells model kidney development and injury. Nat Biotechnol 2015;33:1193–200.*

surprising given the distinct embryologic origins of the nephric duct and the NPCs and the approaches taken to serially differentiate stem cells toward MM. To overcome this developmental barrier, the Nishinakamura group developed an ambitious strategy to coculture UB and NPC tissues that were derived in parallel from mouse ESCs [209,210]. This produced remarkable results that not only led to a branching ureteric epithelium, but also a recreation of the progenitor niche and iterative waves of differentiation that built nephron units within the organoids. The drawbacks of this method include the need for cell enrichment due to a less efficient embryoid body–based differentiation system, less impressive results with human PSCs, and probably most importantly, its dependence on SP cells isolated from fetal kidneys. However, these deficiencies are not insurmountable, as others have recently reported the differentiation of hPSCs into UB tissues [211], and a coculture method will likely be required to fully recapitulate the human kidney development in vitro.

Once stimulated for induction, the NPCs within kidney organoids differentiate in a stochastic manner to produce various nephron structures. While this generates cellular diversity and complexity that may be desired for some applications, when using current protocols there is minimal control in determining which cell types form. Further, the indiscriminate three-dimensional culture conditions lead to appreciable variability between experiments [212]. Thus one's ability to explicitly study or utilize a single cell type is limited by multiple challenges, including but not restricted to, the relative scarcity of any particular cell within an organoid. In this regard, it would be advantageous to identify conditions that specifically promote differentiation directed toward individual nephron lineages. To this end, groups have identified methods to efficiently bias differentiation toward the podocyte fate [213,214],

and the resulting cells have been used to model the filtration barrier and drug-induced glomerular injury. Future efforts to refine the podocyte fate and develop other nephron segments will likely depend on further elucidation of the signaling mechanisms underlying patterning in the developing kidney, further attesting to the important role that developmental biology plays in guiding the addition of exogenous factors or 3D context that predict cell fate in organoids.

The technologies for differentiating renal cell types and organoids from hPSCs have been rapidly advancing over the past several years, and the practical utilization of these tissues is in its infancy. One can envision numerous applications, including disease modeling, physiologic studies, and drug testing or screening, which are already underway. These approaches will also likely be supported by evolving bioengineering techniques, such as bioprinting and organs-on-chip. Further, in vitro hPSC differentiation offers an unprecedented opportunity to model and experimentally manipulate human kidney development, which may help resolve species-specific mechanisms of organogenesis. Eventually, it might be possible to generate tissues for clinical application in kidney regeneration or renal replacement therapy. For example, hPSC-derived early renal progenitors were able to engraft and restore kidney function in a mouse model of AKI [215]. However, generation of an autonomously functioning kidney is a much more ambitious goal that is influenced by several important and substantial obstacles. A therapeutically useful renal tissue construct would require a system for urinary excretion, such as a communicating collecting duct network. In addition, a considerable number of nephrons or "nephron equivalents" are necessary to provide adequate clearance. In vivo, exponential growth in nephron number in the embryo is produced by the elaborate and iterative branching mechanism described in previous sections of this review; however, current hPSC kidney organoids do not exhibit this property. Aside from incorporation of an organized ureteric progenitor population to promote branching, these features could potentially be engineered into organoids by using decellularized kidney scaffolds (reviewed in Ref. [216]). Further, tubules within organoids represent a primitive state of differentiation, analogous to a mid-gestation fetus [204], and our current understanding of maturation mechanisms is extremely limited. However, early evidence is encouraging that organoids undergo significant maturation and development, including vascularization, once transplanted to an in vivo environment [217,218]. Furthermore, it is possible to use the current protocols to carry out toxicity assays or phenocopy some key components of diseases such as cyst formation in polycystic kidney disease, suggesting that they may be useful for testing of therapeutic agents using candidate or nonbiased screening approaches. Nonetheless, additional efforts are required to optimize kidney organoids and unlock their full potential.

Conclusion

There has been remarkable progress in the field of kidney stem cells and regeneration, and recent advances have largely been guided by basic principles of renal embryology. Several strategies exist for efficient derivation of NPCs from hPSCs, as well as other sources (Fig. 44.4),

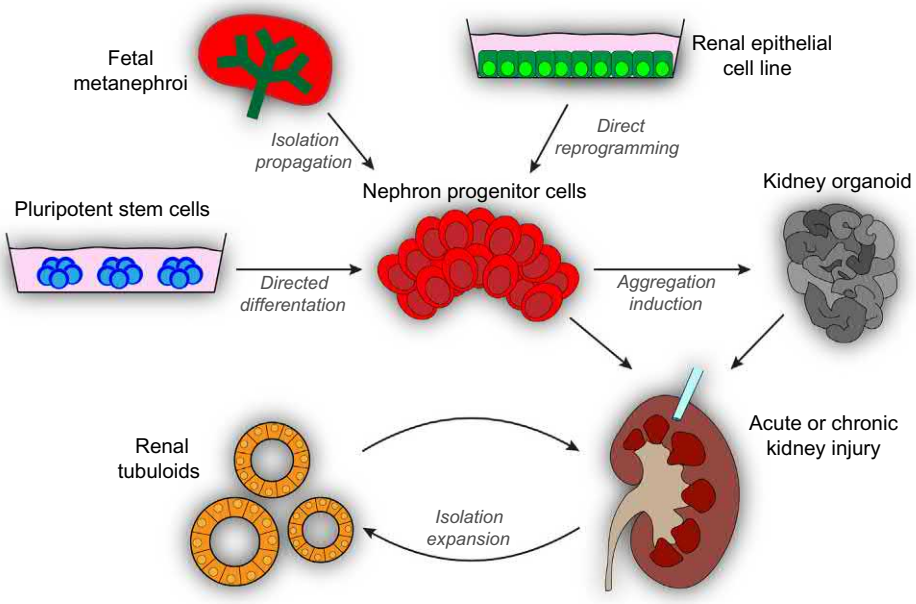

FIGURE 44.4 Putative sources of stem/progenitor cells for potential use in kidney regenerative biology.

which have the capacity to differentiate into numerous nephron cell lineages. Further efforts are now underway to unlock the pathophysiological and therapeutic discovery potential, as well as capacity for clinical renal replacement, of these cells, either through augmentation of endogenous repair processes or via in vitro formation of functional tissues and bioengineered devices. The key obstacles to such endeavors highlight major gaps in our understanding of certain aspects of kidney development, including the growth and maturation of nephron structures and the integration of a sophisticated vascular supply to the organ. The synergistic combination of in vivo and in vitro experimental studies will help further elucidate the developmental and regenerative mechanisms required to promote enhancement of kidney progenitor cells and organoids, ultimately providing strategies for replacement of kidney function.

Disclosures

JVB is cofounder and holds equity in Goldfinch Bio. JVB is co-inventor on KIM-1 patents assigned to Partners Healthcare, received grant funding from Boehringer Ingelheim, holds equity in Dicerna, Goldilocks, Innoviva, Medibeacon, Medssenger, VeriNano, Rubius, SensorKinesis, Sentien, Theravance, and Thrasos and has received consulting income from Biomarin, Aldeyra, Angion, PTC, Praxis, and Sarepta. JVB's interests were reviewed and are managed by BWH and Partners HealthCare in accordance with their conflict of interest policies.

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Chapter 45

Tissue engineering of the kidney

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Introduction

Kidney disease, including acute kidney injury (AKI), chronic kidney disease (CKD) and end-stage renal disease (ESRD), is one of the major worldwide health issues [1]. AKI is defined as a rapid decline in renal function, specifically of blood filtration, resulting in an increase in serum creatinine levels and a decrease in urine output [2,3]. Although AKI is a reversible condition, it often progresses to CKD. CKD is a leading cause of mortality and morbidity in western countries with 8%−16% of the adult population suffering from CKD, characterized by a reduced glomerular filtration rate and increased urinary albumin extraction [4,5]. ESRD is the devastating last stage of CKD that can affect multiorgan systems [6]. The current treatment options for kidney disease include lifelong dialysis and kidney transplantation. Although dialysis replaces renal filtration by removing toxins through extracorporeal blood purification, it cannot fulfill other renal functions essential to maintain health [7]. Kidney transplantation is the only definitive treatment for CKD and ESRD patients. Unfortunately, shortages of transplantable donor kidneys (<20% of the demand) and complications such as immune rejection remain problematic [8].

Tissue engineering and regenerative medicine approaches have been identified as promising solutions to address unmet needs in restoring renal functions. Recent progress in stem cell biology and cell culture technologies has led to the identification and development of reliable and efficient cell sources for the treatment of kidney diseases. These therapeutic cells can be administered through systemic injections to patients or through intrarenal implantations with engineered kidney constructs or carriers. One remarkable achievement, for example, is in the use of autologous mesenchymal stem cells (MSCs) in clinics for the kidney transplantation patients [9−11].

Numerous efforts have been made to bioengineer complex three-dimensional (3D) renal constructs. The kidney is a highly complex organ composed of over 30 different cell types. Each cell type is intricately organized and functionally compartmentalized to form thousands of nephrons, the functional units of the kidney [12]. Therefore to replace such complex renal tissues and restore renal functions, several types of 3D renal constructs have been developed using appropriate cell sources and scaffolding systems [13−15]. The application of engineered 3D renal constructs in preclinical renal failure models has shown the formation of renal structures and restoration of renal functions, such as urine production [15]. The results demonstrate the feasibility of bioengineered renal constructs for augmenting renal function. In addition, recent progress in whole organ engineering associated with decellularization/recellularization techniques opens a new era of whole kidney replacements with tissue-engineered whole kidneys [16−18]. While this approach is still in its infancy and several challenges remain, its shows great potential to fabricate functional whole kidney constructs with complex renal structures and functions with intact vasculature.

Another emerging approach in kidney tissue engineering is in situ tissue regeneration. In situ tissue regeneration is the utilization of the body's own regenerative capability by mobilizing and recruiting host stem or progenitor cells to the injury sites to regenerate or repair injured tissue [19−21]. Different from other cell-based tissue engineering approaches, the in situ tissue regeneration approach has several advantages such as elimination of in vitro cell manipulation processes. To enhance kidney regeneration, several target-specific scaffolding systems [19,22−25] and bioactive factors [19,21,26] have been investigated for efficient endogenous stem or progenitor cell recruitment, functional differentiation, and niche formation [19,22−25].

In this chapter, we will review and discuss the current strategies and therapeutic outcomes that show potential

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for the treatment of kidney diseases, including cell-based engineering of 3D renal constructs, decellularization/recellularization strategy for whole kidney engineering, and in situ kidney regeneration.

Cell-based tissue engineering of the kidney

Recent advances in the fields of cell/stem cell biology and material sciences allow the development of several types of bioengineered cell-based 3D renal tissue constructs, which show therapeutic potential for the reconstruction or repair of damaged kidneys and restoration of normal kidney function [27–29]. In cell-based bioengineering, cells seeded in scaffolds provide biological and anatomical functions to the engineered tissue constructs. The 3D scaffolding systems fabricated by using biomaterials provide suitable microenvironments to the cells and support cell growth, differentiation, maturation into desirable cell types, and integration with host renal tissues. Bioactive molecules, such as growth factors, cytokines, and small molecules may also be incorporated into bioengineered renal tissue constructs to accelerate tissue formation and improve renal function [30]. In this section, we will introduce current updates and discuss the two major components of bioengineered 3D renal tissue constructs, cell sources and biomaterials-based scaffolding systems, as well as preclinical and clinical outcomes for the treatment of kidney diseases.

Cell sources

The adult kidney is composed of over 30 specialized types of cells, which are distributed to various distinct renal compartments. Each cell type is structurally organized into renal vasculature, interstitia, glomeruli, and tubules with architectural complexity and exerts its own functions [31]. Therefore identification and development of reliable and effective cell sources is a prerequisite for kidney tissue engineering. Recent advances in stem cell biology and cell culture techniques have been made to identify, obtain, and culture a number of cell sources for treating kidney diseases (Table 45.1) [72].

Recently, researchers have developed isolation and culture techniques for primary renal cells isolated from both normal and diseases kidney tissues [32,33]. In addition, renal stem or progenitor cells can be obtained from adult kidneys. Although the existence and identification of kidney-derived stem or progenitor cells has been controversial, several studies have reported that renal stem cells can be identified and isolated from the Bowman's capsule, renal papilla, and renal tubules [73]. These findings indicate that the primary renal cells or stem cells derived from patients' own kidneys can be a promising and practical cell source for the treatment of renal diseases. In addition, various types of adult stem/progenitor cells or pluripotent stem cells (PSCs) derived from nonrenal tissues have been discussed as potential cell sources. PSCs, either embryonic stem cells (ESCs) [74,75] or induced PSCs (iPSCs) [76], have been shown to generate organ-level renal tissues with cellular and architectural complexity. However, due to regulatory and ethical issues, and teratoma formation is a major hurdle for clinical applications. Fetal and adult stem cells such as amniotic fluid stem cells (AFSCs) [77] and MSCs [78] possess self-renewal and differentiation capabilities to develop into renal-specific lineages. Fetal and adult stem cells have lower cell growth and tissue formation capability than PSCs, but they are relatively free from ethical issues and teratoma formation, so a number of clinical studies have been performed using fetal and adult stem cells to treat kidney diseases.

Kidney tissue–derived primary or stem/progenitor cells

Primary renal cells

Primary human renal cells can be isolated from normal and diseased kidney tissues and expanded in culture maintaining their phenotype and function. Proximal tubular cells in the native kidney play a critical role in renal functions such as reabsorption of proteins and ions, hydrolase activity, and erythropoietin (EPO) production [79]. The major cell population of the normal kidney is the proximal tubular cell, comprising about 60% of total cell population, including podocytes, distal tubular cells, descending loop of Henle, and collecting duct cells [80]. Thus primary proximal tubular cells with normal physiology isolated from human kidneys are a powerful renal cell source for autologous cell-based treatments. However, primary renal cells have limited cell proliferation capacities of up to five passages in in vitro culture, highlighting the need for more reliable and reproducible cell culture systems [81,82].

Recently, George et al. focused on the isolation and expansion of primary human renal cells from normal kidneys and from diseased kidneys of CKD patients for the potential use in clinical applications [32,33]. The expanded primary renal cells showed efficient and prolonged proliferation with no differences in growth patterns until passage 9. The majority of the primary renal cells were proximal tubular cells (approximately 70%–80%) with the remaining cells consisting of distal tubular cells, collecting duct cells, podocytes, and other cell types. In 3D culture, primary renal cells formed tubule-like structures with functional properties. Remarkably, primary renal cells from human kidneys of CKD patients had normal phenotypic and functional characteristics similar to the cells from normal kidneys [33]. This study

TABLE 45.1 Cell sources for engineering of the kidney.

Cell type	Origin	Outcome	Refs.
Primary renal cells			
Unpurified human primary renal cells	Human normal and diseased kidneys	High percentage of proximal tubular cells (>80%), high proliferation capability up to passage 9, tubule-like structures with functional properties	[32,33]
EPO-enriched cells	Purified human renal primary cells	Structural and functional improvement in ischemic injury models in rats, amelioration of inflammation and oxidative stress	[34]
Human $CD10^+CD13^+$ cells	Purified proximal tubular epithelial cells	Maintenance of phenotypes and functions over several passages	[35]
Stem/progenitor cells derived from renal tissue			
Human $CD133^+$ cells	Bowman's capsule	Homing and integration with host tubules in a mouse tubulonecrosis model	[36]
Human $C24^+CD133^+$ cells	Bowman's capsule	Regeneration of renal tubules and amelioration of renal function in a mouse acute renal failure model	[37]
Human $CD133^+CD24^+PDX^-$ cells	Bowman's capsule	Regeneration of both tubules and podocytes, reduced proteinuria and glomerular damages in a mouse acute renal failure model	[38]
LRCs	Papilla	Heterogeneity, epithelial, mesenchymal, and neuronal phenotypes; contribution to kidney repair in response to renal ischemic injury	[39,40]
$CD133/1^+$ $nestin^+$ cells	Papilla	Integration and tubule formation	[41]
BrdU retaining and $vimentin^+$ cells	Tubules (S3 segment)	Integration and regeneration of renal tubules in an ischemic injury model	[42]
rKS53	Tubules (S3 segment)	Replacement of injured tubular cells and differentiation into mature tubular epithelial cells in rat AKI models	[43]
$ALDH^{high}$ cells	Proximal tubules	$CD133^+CD24^+$, increased cell proliferation	[44]
Adult stem cells			
BM-MSC	Bone marrow	Functional improvement in mouse and rat models, amelioration of renal transplantation–related complications in human clinical trials	[9–11,45–52]
AD-MSC	Fat tissue	Functional improvement in mouse, rat, and pig models	[53–57]
Fetal stem cells			
AFSC	Amniotic fluids	Functional improvement in mouse renal failure models and pig kidney transplantation models	[58–61]
Pluripotent stem cells			
ESC	Embryo	In vitro differentiation into renal population, ESC-derived cells integration into proximal tubules	[62–65]
iPSC	Genetically modified somatic cells	In vitro differentiation into renal population, functional improvement in a rodent AKI models	[66–71]

AD-MSC, Adipose-derived mesenchymal stem cell; *AFSC*, amniotic fluid stem cells; *AKI*, acute kidney injury; *ALDH*, aldehyde dehydrogenase; *BM-MSC*, bone marrow–derived mesenchymal stem cell; *BrdU*, 6-bromo-2′-deoxyuridine; *EPO*, erythropoietin; *ESC*, embryonic stem cells; *iPSC*, induced pluripotent stem cells; *LRC*, label retaining cells; *PDX*, podocalyxin.

indicates that renal cells isolated from diseased kidneys can be a reliable autologous cell source to treat patients with renal failure.

Thereafter, they purified an EPO-enriched cell population from cultured primary kidney cells and investigated the therapeutic potential of these cells using a rat CKD

model [34]. Intrarenal injection of EPO-enriched cells was capable of functional improvements such as amelioration of inflammation and oxidative stress compared with unpurified cells. These results suggest that EPO-enriched cells within primary kidney cells may be a promising cell source for the treatment of renal failure.

Proximal tubular epithelial cells can be further purified from human renal tissue [35]. $CD10^+CD13^+$ proximal tubular cells exist in very low population densities of 4%. However, once purified, the cells proliferated and maintained phenotypic and functional properties over several passages. In another study, human renal epithelial cells from human thick ascending limbs and early distal tubules were isolated using an immunomagnetic cell isolation technique, showing that these cells can be used as a cell source for an in vitro system and for treating the renal diseases targeting human thick ascending limb of Henle's loop and early distal tubules [83].

Stem/progenitor cells derived from kidneys

Renal stem cells in Bowman's capsule The primary function of the kidney is to remove waste and generate urine by filtrating blood from the glomerulus into the Bowman's capsule [84]. In the filtration process, podocytes, a specialized type of epithelial cell, play a key role; however, podocytes have very limited proliferation in the damaged kidney [85]. Therefore several attempts have been made to identify and obtain a source of renal stem cells capable of replacing podocytes to restore normal kidney filtration functions.

It is believed that $CD133^+$ or $CD133^+CD24^+$ cell, a subset of parietal epithelial cells in the Bowman's capsule, is a possible adult stem cell source. A large population of $CD133^+$ cells were observed in developing embryonic kidneys with self-renewing and multipotent characteristics, and $CD133^+$ cells remained scattered through the tubules and the urinary pole of Bowman's capsule in the adult kidneys [37,38,86]. $CD133^+$ or $CD133^+CD24^+$ renal stem cells can be isolated and expanded from the normal adult kidney cortex and in the kidneys of cancer patients [36,37]. $CD133^+CD24^+$ cells isolated from Bowman's capsule were positive for the stem cell markers CD106, CD105, CD54, and CD44, but negative for podocyte markers, indicating that these cells were not fully differentiated into renal lineages [37,38]. $CD24^+CD133^+$ cells could differentiate into renal lineages, including mature proximal and distal tubules in in vitro culture conditions, showing their possible use as a cell source for kidney regeneration [37].

Due to their multipotency, especially their renal-specific lineage differentiation capabilities, $CD133^+$ cells have been applied to treat renal disease, showing therapeutic potential in in vivo studies. In a study done by Bussolati et al., subcutaneously implanted $CD133^+$ cells in mice formed tubular structures and functional vessels, and intravenously injected $CD133^+$ cells in mice with glycol-induced tubular necrosis successfully homed to and integrated into host tubules [36]. In another study, $CD24^+CD133^+$ cells injected into mice acute renal failure models demonstrated therapeutic potential, evidenced by the regeneration of renal tubules and amelioration of renal function [37]. In addition, injection of $CD24^+CD133^+$ podocalyxin $(PDX)^-$ cells, but not $CD24^+CD133^+PDX^+$ cells, regenerated both tubular cells and podocytes and reduced proteinuria and glomerular damage [38].

Renal stem cells in the papilla The papilla is the apex of the renal pyramids that projects into the lumen of a calyx of the kidney. The papilla contains the epithelial cells of the collecting ducts and the loops of Henle where urine flows into the ureter. Renal stem cells also reside in the papilla. The presence of a stem cell population in the papilla was identified by Oliver et al. [39,40]. Using a nucleotide bromodeoxyuridine (BrdU) labeling technique, they demonstrated that the renal papilla contained BrdU label retaining cells (LRCs) in rodents. The LRCs isolated and cultured in vitro showed a heterogeneous population of ZO-1^+ epithelial, α-smooth muscle $actin^+$ mesenchymal, and β-III $tubulin^+$ neuronal phenotypes. An in vivo study indicated that LRCs contributed to kidney repair in response to renal ischemic injury.

$CD133^+$ cells can be obtained not only from the Bowman's capsule as described earlier [36] but also from the renal papilla [41] in human kidney tissues. Human $CD133^+/1^+$ renal papillary cells were $nestin^+$ and also positive for human ESC (hESC) markers SSEA4, Nanog, SOX2, and OCT4/POU5F1. These cells integrated into developing kidney tubular structures indicating their capability to form renal tubules de novo.

Renal stem cells in tubules The renal tubules play a critical role in the reabsorption of proteins, water, and electrolytes in filtrated fluids. The S3 segment of the proximal tubules has been investigated to identify and isolate renal stem cells in several studies [42,87]. The S3 segment is located near the corticomedullary junction and therefore incurs more severe damage from ischemic injuries than other segments of the nephron. As such, a number of stem cells expressing vimentin and proliferating cell nuclear antigen have been found to reside to repair the damages in the S3 segment [42,87,88]. More recent work reported that renal tubular cells with high aldehyde dehydrogenase (ALDH) activity ($ALDH^{high}$ cells) can be isolated from human tubule tissues [44]. The $ALDH^{high}$ cells showed $CD133^+CD24^+$ phenotypes and had improved cell proliferation capabilities compared to

$ALDH^{low}$ cells. The result indicates that $ALDH^{high}$ cells are renal progenitor cells in adult human tubules.

There were several reports that stem cell populations isolated from renal tubules formed regenerating renal tubules in the ischemia/reperfusion injury model in mice [89–91]. In a study done by Kitamura et al., an epithelial-like cell line, rKS53, was established by purifying cells with a high proliferation rate from the microdissected S3 tubules of rats [43]. This cell line has stem/progenitor cell–like features and expresses the stem cell markers vimentin, c-Met, Sca-1, c-kit, Pax-2, and the neural stem cell marker, Musashi-1, in addition to having the potential to differentiate into different tubule phenotypes. When the rKS53 cells were injected into ischemic kidneys, the cells could replace injured tubular cells and differentiate into mature tubular epithelial cells, demonstrating the regenerative capabilities of rKS53 cells.

Adult and fetal stem cells

Mesenchymal stem cells

MSCs isolated from bone marrow (BM) and fat tissues have been extensively utilized as a promising cell source for the treatment of renal diseases in preclinical and clinical settings [78]. MSCs are easily isolated through minimally invasive techniques such as BM extraction (BM-derived MSCs, BM-MSCs) and liposuction (adipose-derived MSCs, AD-MSCs), expanded with high efficiency in culture, and relatively free from ethical issues compared to the other cell sources. With their self-renewal capabilities and multipotency, BM-MSCs [45–52] and AD-MSCs [53–57] have been applied to regenerate several injured organs, including kidneys [78,92].

Numerous studies demonstrated the therapeutic potential of MSCs to treat renal failure; however, the mechanism of repair and regeneration of the damaged kidney by MSCs treatments is still controversial. Early studies reported that transplanted MSCs differentiate into renal-specific lineages, such as renal tubular cells [90], podocytes [52], glomerular cells [93], and mesangial cells [94], and integrate into host renal tissues to improve renal structure and function in AKI models. On the other hand, recent studies suggest that the predominant mechanism of kidney regeneration by MSCs in AKI models is through their paracrine effects. Trophic factors or secretomes released from transplanted MSCs allow endogenous cells to proliferate and prevent apoptosis to accelerate kidney repair [95,96]. The paracrine effects of MSCs on kidney repair have also been demonstrated in the several studies using MSC-conditioned media [97].

To date, a number of clinical studies have been performed using autologous MSCs for the success and amelioration of kidney transplantation–related complications because of MSCs' low immunomodulatory effects and high regenerative capability [9–11]. For instance, a safety and feasibility study demonstrated that the infusion of autologous MSCs to kidney recipients controls $CD8^+$ T cell function and reduces immune rejections [10]. In another study, autologous BM-MSCs were injected into kidney transplant patients [11]. With 6-month follow-up, the autologous BM-MSC injections improved the resolution of interstitial fibrosis/tubular atrophy. Recently, there are some efforts to initiate Phase I/II clinical trials for restoration of renal function in renal failure patients using autologous MSCs [5]. Despite the promising results of using MSCs for the treatment of renal failure, side effects such as increased interstitial fibrosis have also been reported [98]. Therefore further safety and feasibility studies are necessary for the successful development of MSC-based therapies for kidney repair.

Amniotic fluid stem cells

AFSCs are another potential cell source for the treatment of renal failure. Recently, human AFSCs (hAFSCs) were isolated from amniotic fluid and cultured in vitro [99]. Isolated AFSCs retained high self-renewal potential, high proliferation rats over 250 population doubling, and multidifferentiation ability, with not reported teratoma formation in vivo. Of importance here was the demonstration of the differentiation capability of AFSCs into renal-specific lineages [77].

Recent preclinical studies using AFSCs demonstrated their possible therapeutic effects in treating kidney diseases [58–61]. Hauser et al. reported that intravenous infusion of hAFSCs showed more rapid recovery of kidney function in an acute renal failure model compared to treatment with BM-MSCs [58]. In another study, preconditioning of hAFSCs with neurotrophic factors achieved enhanced renal function outcomes compared to the use of hAFSCs without preconditioning in an AKI model [59]. In addition, injection of AFSCs prevented renal fibrosis and preserved renal function in a mouse CKD model [60] and a porcine model of kidney transplantation [61].

Pluripotent stem cells

Embryonic stem cells

The representative PSCs are ESCs [74,75] and iPSCs [76]. Both ESCs and iPSCs are capable of self-renewal and multidifferentiation into all cell types, cells of mesodermal, endodermal, and ectodermal lineages. Since it has been demonstrated that hESCs can be obtained from the inner cell mass of the embryonic blastocyst, hESCs represented a powerful cell source for developing in vitro models and for treating damaged tissues [75,100,101]. Extensive research has been conducted to establish robust protocols for hESC differentiation to produce functional cells of desired lineages.

Differentiation of ESCs into renal lineages has also been investigated [62–65]. For instance, Kim and Dressler reported that mouse ESCs could be differentiated into renal epithelial cells using nephrogenic growth factors and that these epithelial cells were efficiently integrated into a developing kidney [63]. Vigneau et al. isolated and purified renal proximal tubular progenitor cells from the embryoid bodies using a defined differentiation protocol. When the proximal tubular progenitor cells were injected into a developing newborn mouse kidney, the cells integrated into proximal tubules that had normal morphology and function, without teratoma formation [65].

These previous results strongly support the feasibility of applying ESCs to treat renal failure. However, the widespread use of ESCs is still limited due to legal and ethical issues associated with the use of human embryos in the clinics, uncontrolled growth and differentiation, possibility of teratoma formation in vivo, and immune rejection after transplantation [102,103].

Induced pluripotent stem cells

Another cell source possessing pluripotency is iPSCs. Unlike ESCs, iPSCs can be obtained from somatic donor cells, such as patient's own fibroblasts [76]. iPSCs were first developed by Takahashi and Yamanaka by reprograming human fibroblasts with four genes, Oct3/4, Sox2, c-Myc, and Klf4 [76]. The genetically modified iPSCs have very similar characteristics to ESCs such as self-renewal, ESCs-specific genes expression, embryonic body formation, and teratoma formation. However, iPSCs are relatively free from ethical issues and low immune rejection complications compared with ESCs. Therefore iPSCs can be utilized as an autologous cell source for the treatment of renal diseases in a clinical setting.

Great progress has been made in recent years to establish iPSC culture techniques and differentiation protocols for renal cell lineages in vitro [66–68]. Several preclinical studies have demonstrated the feasibility of iPSC-derived cells to treat kidney diseases [69–71]. For instance, Song et al. reported that the direct differentiation of human iPSCs (hiPSCs) into podocytes was possible and that these hiPSCs-derived podocytes were integrated into metanephric kidney structures [67]. In another study, human kidney organoids containing multiple lineages were generated from hESCs and iPSCs in a 3D culture using a growth factor, FGF9, and a small molecule, CHIR99021 [68]. The kidney organoids contained functional nephrons that were composed of glomeruli with podocytes, distal and proximal tubules, early loops of Henle, collecting ducts, renal interstitium, and endothelial cells. This protocol was modified to a two-dimensional (2D) culture method to generate kidney progenitor cells that were subcutaneously implanted into mice [104]. The implanted kidney progenitor cells formed kidney-like structures composed of glomeruli, tubules, mesangial cells, and capillaries after 3 months. Remarkably, a few following studies demonstrated that the transplantation of renal progenitors differentiated from hiPSCs via intravenous injection [70] or renal subcapsular transplantation [71] has therapeutic effectiveness, attenuating histopathological changes and restoring renal functions.

Despite these promising results, several concerns remain for further clinical use of iPSCs as a cell source. It is difficult to use iPSCs due to multistep differentiation processes that are specifically timed and utilize highly sophisticated bioactive molecules [105,106]. The low efficiency and unpredictability of differentiation in vivo are other challenges to using iPSCs. Poorly differentiated metanephric tissues and islands of cartilage formation have been frequently observed after transplantation [104]. The immunogenicity of the differentiated cells needs to be carefully accessed [107].

Tissue-engineered cellular three-dimensional renal constructs

Engineering three-dimensional kidney constructs using natural and synthetic polymers

Numerous tissue-engineered cellular 3D kidney constructs have been developed using a variety of scaffolding systems, fabricated by natural or synthetic polymers for treating renal diseases (Table 45.2). The basic strategy of engineering renal constructs is to seed cells onto scaffolds with desirable biological, mechanical, or structural properties to support cellular behavior and functions such as proliferation, differentiation, integration, and kidney-like tissue formation. Therefore the ideal scaffolding system is biocompatible: it has porous structures to provide a suitable space with efficient oxygen and nutrients to the cells for survival, provides appropriate biological microenvironments for growth and maturation to desired tissues, possesses the appropriate mechanical properties to support tissue formation and integration of the engineered constructs in vivo, and has appropriate biodegradation rates to allow the cells to produce their own extracellular matrix (ECM) that will eventually replace the scaffolds with newly formed tissues [114].

Natural polymers or synthetic polymers have been widely used as biomaterials to engineer 3D renal constructs. Natural polymers include collagen, hyaluronic acid, fibrin, gelatin, alginate, agarose, and chitosan. Most of the naturally derived polymers are biocompatible and biologically active, and therefore capable of supporting cell adhesion, proliferation, differentiation, and migration.

TABLE 45.2 **Cell-based three-dimensional (3D) renal constructs using biomaterials.**

Biomaterials	Cells	Outcome	Refs.
Natural polymers			
Collagen/ vitrigel	Glomerular epithelial and mesangial cells	Reconstruction of 3D renal glomerular tissue in vitro	[108]
Collagen/ Matrigel	Neonatal rat renal cells	Casting mold system as static stretching device, self-assembly of renal cells tubules and glomeruli-like structures formation with reduced necrosis in vitro	[109]
Collagen	Human primary renal cells	Tubulogenesis and function (albumin adsorption) in vitro cells survival for up to 6 weeks within the kidney in vivo	[32]
Collagen/PCL	Human endothelial cells	Perfusable and endothelialized biomimetic vascular scaffolds for 3D renal tissue constructs	[13]
HA	Fetal kidney tissue	Renal tubules and glomerular structures formation in vitro	[110]
Gelatin/fibrin	Human proximal tubule epithelial cells	Engineering of 3D proximal tubule on a chip using a 3D bioprinting technology, enhanced epithelial morphology and function compared to the cells in 2D culture	[14]
Synthetic polymers			
PGA	Rat renal segments	Nonwoven fibrous scaffolds, glomeruli and tubules formation in a subcutaneous implantation in mice	[111]
PCL	Human kidney primary epithelial cell line	Electrospun scaffold fabrication with high porosity, cell infiltration throughout the scaffold	[112]
PC	Mouse primary renal cells	Tubular-shaped construct formation, yellow urine–like fluid production, glomeruli and highly organized tubule-like structures formation, and vascularization in subcutaneous implantation in mice	[113]
PGA, PC, PE	Renal cells cloned from bovine fibroblasts	Kidney-like unit, urine-like fluid production, unidirectional secretion and concentration of urea nitrogen and creatinine, and formation of organized glomeruli- and tubule-like structures in vivo	[15]

2D, Two-dimensional; *3D*, three-dimensional; *HA*, hyaluronic acid; *PC*, polycarbonate; *PCL*, polycaprolactone; *PE*, polyethylene; *PGA*, polyglycolic acid.

However, natural polymers have poor mechanical properties compared to synthetic polymers. Even so, natural polymer–based scaffolds continue to be widely used for kidney tissue engineering due to their favorable biological properties [115].

Unlike natural polymers, synthetic polymers such as polylactic acid, polyglycolic acid (PGA), and polylactic-*co*-glycolic acid can be synthesized with tailored architecture, so it is easy to control their mechanical properties and degradation characteristics for several weeks to several years. However, synthetic polymers are less biocompatible than natural polymers and their degradation products may stimulate inflammatory responses [116]. Therefore a variety of alternative composite scaffolds combining synthetic polymers with natural polymers have been investigated.

Collagen is a one of the major structural components of the ECM. Collagen as a biomaterial is inherently biocompatible and degradable and has been extensively utilized to fabricate tissue-engineered renal constructs [117]. Numerous reports have demonstrated that collagen is able to provide 3D microenvironments to support cell attachment and growth. For instance, Wang and Takezawa [108] developed a novel technique of renal cell isolation and a long-term 3D culture system using a collagen-based scaffolding system for the reconstruction of renal glomerular tissues in vitro. Glomerular epithelial and mesangial cells were isolated from kidney tissue and cultured on a collagen–vitrigel scaffold. The collagen–vitrigel scaffold provided a suitable environment similar to the glomerular basement membrane for the cells and resulted in the maintenance of cell growth and viability over 1 month, and reconstitution of renal glomerular tissues. In another study, Guimaraes-Souza et al. demonstrated that human primary renal cells could reconstitute the functional renal tissue in vitro using a 3D collagen scaffold [32]. Primary renal cells were differentiated into tubule-like structures composed of proximal tubules, distal tubules, and collecting ducts showing functionality such as albumin uptake. The engineered renal constructs were then implanted into the kidney parenchyma, showing cell survival for up to 6 weeks and integration into glomeruli and interstitium.

These results show that collagen-based tissue-engineered 3D renal constructs utilizing the patients' own cells may be used for restoration of renal function.

While natural polymer–based scaffolds have been widely utilized for tissue regeneration, they have low mechanical properties and sometimes fail to retain shape as described earlier. Natural polymer–based hydrogels contract over time with growth and proliferation of the seeded cells leading to cell death in the center. To prevent scaffold contraction and improve cell viability, Lu et al. utilized a 3D collagen/Matrigel scaffold with casting molds to provide static stretch for neonatal rat renal cell culture. Using this system, neonatal rat renal cells self-assembled into renal tubules and glomeruli-like structures with reduced necrosis [109].

The mechanical properties and degradation rates of synthetic polymers are relatively easy to control and process for the purposes of scaffold fabrication. Therefore synthetic polymers have been more frequently applied to fabricate scaffolds that need more controlled complex architecture than natural polymers. For instance, Burton et al. developed electrospun scaffolds using polycaprolactone (PCL) using a cryogenic electrospinning technique [112]. The resulting scaffolds provided fibrous 3D structures with higher porosity, facilitating cell infiltration throughout scaffolds compared to random or aligned electrospun scaffolds. They also state that the architectural features of the scaffold, including fiber alignment and diameter, affected the attachment, viability, and alignment of renal cells emphasizing the importance of the scaffold architecture on kidney regeneration. Another study by Kim et al. utilized a biodegradable, nonwoven PGA fiber mesh (12 μm in diameters) as a scaffold in order to culture renal segments [111]. They isolated renal segments from rat kidneys, which included nephron epithelial cells, endothelial cells, vascular smooth muscle cells, and stromal cells. An in vivo study of subcutaneous implantation of the renal segment-seeded PGA mesh in mice showed that the 3D renal constructs formed glomeruli and tubules, demonstrating the possibility of reconstituting renal structures using fibrous polymer scaffolds.

Yoo et al. developed a functional artificial renal unit that exhibited urine production [113]. The artificial renal unit was fabricated by seeding mouse renal cells in tubular-shaped polycarbonate scaffolds. This renal unit formed glomeruli and highly organized tubule-like architecture upon subcutaneous implantation in mice. Remarkably, the newly formed renal tissues excreted yellow urine–like fluid. This renal unit was further advanced in a following study by Lonza et al. Their renal unit was composed of cell-seeded unwoven PGA scaffolds and collecting systems constructed by three cylindrical polycarbonate membranes, which were connected to a polyethylene reservoir. The renal unit self-assembled into glomeruli- and tubule-like structures with continuity and showed kidney-like functions such as the production of urine-like fluids and unidirectional secretion of urea, nitrogen, and creatinine in vivo.

With an advanced scaffold fabrication technique, several attempts have made to produce 3D renal scaffolds that mimic native renal structures. For instance, Huling et al. developed a biomimetic vascular scaffold for a 3D renal tissue construct using a novel vascular corrosion cast technique [13]. Vascularization is among the most challenging aspects in fabricating engineered tissues for maintaining viability and function of cells in vitro and in vivo [118]. Also, kidney is a highly vascularized organ, and the very sophisticated renal vasculature plays a critical role in cell viability as well as renal function in blood filtration. In this study, native renal microvasculature was captured using PCL as a sacrificed template followed by embedding into collagen. The resulting collagen scaffolds possessed normal kidney microvasculature and were capable of endothelialization and perfusion, showing potential for application in kidney tissue engineering.

Advances in microfluidics in the last decade and cell culture have made micro-engineered models of the functional units of several human organs, so-called organ-on-a-chip [119,120]. The bioengineered kidney-on-a-chip was fabricated using several types of synthetic and natural polymers where cells were seeded to mimic the functional unit of the kidney. In one study by Homan et al., an engineered 3D proximal tubule-on-a-chip was fabricated by using 3D bioprinting technology [14]. 3D bioprinting is an emerging scaffold fabrication technique that is suitable for creating complex and luminal tissue structures [121]. In the study of Homan et al., 3D printed tubular structures were embedded within ECM and housed in perfusable tissue chips, and then the tubular lumen was seeded with proximal tubule epithelial cells. The resulting engineered 3D proximal tubules-on-a-chip showed enhanced epithelial morphology and function compared to the cells in 2D culture with response to nephrotoxin. Therefore the engineered 3D proximal tubules-on-a-chip can be applicable for in vitro models in studying renal physiology, disease modeling, and toxicity testing.

Decellularization/recellularization strategy

Recently, there have been numerous efforts to bioengineer complex 3D organs and tissues such as the heart, liver, lung, and kidney. Such organs have tissue-specific multicellular architecture and vasculature that is sophisticatedly organized to exert tissue-specific functions. Kidney, as mentioned earlier, is a highly complex organ composed of over 30 different cell types. Each cell type is intricately organized and functionally compartmentalized to form thousands of nephrons, the functional units of the kidney.

Nephrons have different regions: the Bowman's capsule that encloses the glomerulus, the proximal tubule, the loop of Henle, the distal tubule, and the collecting duct. Each region has different anatomical features and physiological roles [12]. This complexity of the kidney makes it difficult to reproduce by traditional scaffold fabrication methodologies.

Recent progress in whole organ engineering, involving the decellularization/recellularization technologies, has provided a promising approach to overcome the limitations of traditional scaffold fabrication techniques and to build complex 3D kidney constructs (Table 45.3) [16–18]. Potentially, this technology may address the unmet medical problem of the shortage of transplantable donor kidneys via an alternative engineered whole kidney. In the decellularization/recellularization approach, acellular tissue is utilized as a scaffolding system. Acellular biologic scaffolds can be produced by removing cells from the tissues or organs through the "decellularization" process, leaving behind a tissue-specific ECM. The decellularized scaffolds can be seeded with cells, in a process called "recellularization" [17,132]. The decellularized tissues maintain the 3D ultrastructure and composition of the ECM, biochemical and biophysical factors, and vascular networks of the native tissues or organs. Over time, the decellularized tissue is degraded and remodeled with new ECM proteins produced by seeded cells. Therefore decellularizing tissues can produce an ideal scaffolding system to provide renal-specific microenvironments for engineering functional whole kidneys.

A variety of decellularization strategies have been developed to generate acellular renal scaffolds using rat [122,123,128–130], pig [124,131], rhesus monkey [126,127], and human [125] kidneys. An efficient decellularization technique is to remove the cellular components to avoid the induction of an immune response while preserving the 3D renal ECM architecture for glomerular and tubular structures and intact vascular networks. The general decellularization protocol for this approach is to perfuse detergents, enzymes, or other cell lysates such as sodium dodecyl sulfate, Triton X-100, and DNase through the inherent vasculature [17,132].

Initial success in kidney decellularization was achieved by Ross et al. in their work on rat whole kidneys [122,123]. Their decellularized rat kidneys preserved the intricate ECM architecture with intact vasculature. They then showed the possibility of using the decellularized kidney as a renal scaffold. They recellularized the acellular kidney scaffold with murine ESCs by manual injection through either the artery or ureter, showing the repopulation and differentiation of ESCs in the acellular kidney scaffolds. Thereafter, several groups have reported efficient decellularization technologies of whole kidneys in pig [131] and monkey [125,127] models to produce acellular renal scaffolds to recreate engineered whole kidney constructs on a clinical scale. Recently, Orlando et al. reported successful decellularization of human kidneys discarded from transplantation with an optimized decellularization protocol [125]. The resulting human whole kidney ECM scaffolds maintained their macro- and micro-3D architecture, biochemical properties, and vascular patency, showing the possibility of utilizing discarded human kidneys for acellular renal scaffolds in whole kidney transplantation.

There have been several attempts to recellularize decellularized whole kidneys to produce functional tissue or organs for transplantation. Early recellularization methods involved manual injection of cells through the renal artery and vein in static culture for several days; however, this approach results in low survival and growth, significant apoptosis, and an uneven distribution of cells [16]. Therefore an efficient recellularization strategy should be established for bioengineering implantable whole kidneys. Recently, a bioreactor system composed of a cell infusion and perfusion culture system such as syringe pump [133], peristaltic pump [130], or pulsatile pump [18,38] has been applied for this process. The bioreactor system allows for constant infusion of cells without apparent damage to the scaffold and supports nutrition, viability, proliferation, and differentiation of the cells within the scaffold [16].

In a recent report by Song et al., a bioengineered rat kidney construct was produced by decellularization/recellularization techniques using a bioreactor system and transplanted into rats [130]. A decellularized rat whole kidney was recellularized using human umbilical vein endothelial cells through the renal artery, and rat neonatal cells through the ureter with negative pressure on the whole kidney chamber, followed by arterial perfusion culture in a bioreactor as previously mentioned. Using this system, they achieved a high recellularization rate, with 70% glomeruli. In another study by Peloso et al., an acellular kidney was repopulated in a customized bioreactor system [129]. In this study, human pancreatic carcinoma cells were manually injected into decellularized rat kidneys through renal arterial vascular networks and cultured in a pulsatile system. In a short-term culture system, the cells were homogenously distributed inside the parenchyma.

Even with the success of these studies, the appropriate source of cells for recellularization is controversial. Current studies use different cell sources, including neonatal kidney cells [130], ESCs [122,123,126–128], primary human renal cells [124], and endothelial cells [130,131], to repopulate acellular kidney scaffolds. ESCs show superior outcomes in terms of differentiation into multiple types of renal cells in bioengineered kidney tissues; however, the use of ESCs in the clinic is still limited due to regulatory and ethical issues. Primary human renal

TABLE 45.3 Decellularization/recellularization strategy for whole kidney tissue engineering.

Species	Decellularization	Recellularization			In vivo	Outcome	Refs.
		Cells	Seeding method	Scaffold culture method			
Rat	3% Triton X-10, DNase, 3% Triton X-100, 4% SDS. Gravity-based perfusion using renal artery and ureter (100 mmHg)	Murine ESCs	Manual injection through either the artery or ureter	Automated perfusion system (120 mmHg/ 80 mmHg)	–	Proliferation and differentiation of ESCs in a complex architecture, production of basement membrane	[122,123]
Pig	0.5% SDS for 36 h and DNase overnight, renal artery perfusion	Human primary renal cells	Static seeding	Static culture for 3–4 days	–	Optimization of decellularization method	[124]
Human	Distilled water at 12 mL/min for 12 h, 0.5% SDS at 12 mL/min for 48 h, and final rinse with PBS at 6 mL/ min for 5 days	–	–	–	–	Optimization of decellularization methodAngiogenic capability	[125]
Rhesus monkey	1% SDS, 7–10 days	hESCs	Static seeding into decellularized kidney sections	Static culture for 8 days	–	Spatial organization cells into tissue-specific structures in vitro	[126,127]
Rat	0.01 M PBS at 2 mL/min for 15 min, 0.5% SDS for 4 h, then PBS for 24 h, renal artery perfusion	Mouse ESCs	Manual seeding through the renal artery and ureter	Perfusion culture	Orthotopic implantation into rat	Reperfusion and urine production with no blood leakage, obstruction of renal artery and vein by a massive thrombi	[128]
Rat	1% Triton X-100 at 70 mL/h for 1 h 25 min, then PBS at 50 mL/h for 1 h	Human pancreatic carcinoma cells	Manual injection through renal arterial vascular network	Perfusion bioreactor at 1 mL/min for 24 h	Orthotopic implantation into rat	Homogeneous cell distribution, obstruction of all vascular structures with thrombi	[129]
Rat	1% SDS 12 h, 1% Triton X-100 30 min, renal artery perfusion (40 mmHg)	Rat neonatal kidney cells and HUVECs	HUVECs: Arterial flow (1 m/min) then static culture overnight, rat neonatal kidney cells: injected through the ureter	Arterial perfusion culture in a bioreactor (1.5 mL/min)	Orthotopic implantation into rat	Graft perfusion by recipient's circulation, urine production through ureteral conduit	[130]
Pig	0.5% SDS	GFP-labeled endothelial cells (MS-1)	Static seeding followed by ramping perfusion, conjugation of CD31 antibodies to the vascular matrix	Perfusion rate at 2 mL/ min and then gradually increased to 5, 10, and 20 mL/min at 10–12 intervals	Orthotopic implantation into pig	Improved endothelial cell retention on the vasculature, enhanced vascular patency of the implanted kidney in vivo	[131]

ESC, Embryonic stem cell; *GFP*, green fluorescent protein; *hESC*, human embryonic stem cell; *HUVEC*, human umbilical vein endothelial cell; *PBS*, phosphate buffered saline; *SDS*, sodium dodecyl sulfate.

cells or renal cells derived from hiPSCs, therefore, may be better candidates when considering clinical perspectives.

Although the decellularization/recellularization strategy has shown promising results in vitro and in vivo, several challenges need to be addressed for the successful transplantation of bioengineered whole kidneys. One of the critical challenges is the maintenance of long-term vascular patency of the bioengineered kidneys in vivo [128,129,131]. In a study by Peloso et al., mentioned earlier, it was reported that their implanted whole rat kidney was able to maintain blood perfusion after orthotopic transplantation for 7 days; however, the entire vascular network for the implant was obstructed by a massive thrombosis over time [129]. With this result, they highlighted that the reendothelialization of scaffold vasculature is mandatory for avoiding extensive thrombosis to establish successful transplantation and renal function. To address this issue, Ko et al. developed a novel endothelial seeding method that facilitated effective reendothelialization of a porcine kidney scaffold [131]. Conjugating CD31 antibodies to the vascular lumen increased endothelial cell attachment and retention on the vascular network, and resulted in reduced thrombosis and enhanced vascular patency of the bioengineered whole kidney in vivo.

Cell-free tissue engineering of the kidney

In situ kidney regeneration

Recent progress in tissue engineering and regenerative medicine has adopted the concept of in situ tissue regeneration. Even though cell-based approaches have shown positive effects on tissue regeneration, this approach is challenged by the difficulties in obtaining appropriate cell sources and achieving higher cell survival rates [118,134]. In situ tissue regeneration is the utilization of the body's own regenerative capabilities, without exogenous cell transplantations, to restore organ function. This approach has several advantages compared to cell-based tissue engineering approaches [19,21]. In situ tissue regeneration does not require cell manipulation ex vivo, such as isolation and expansion of cells in large quantities from tissue biopsies. Instead, target-specific scaffolding systems and/or delivery of bioactive molecules can be applied to provide an appropriate niche for enhancing the ability of tissue regeneration [19,22–25]. In addition, considering that current cell-based tissue engineering techniques still struggle to create functional 3D tissues with the structural and cellular complexities of the kidney, in situ tissue regeneration may be more clinically feasible approach.

The simplest and most pharmacologically attractive strategy for in situ tissue regeneration is the delivery of bioactive molecules, such as growth factors, chemokines, or small molecules that support the kidney's own regenerative capability for proliferation, functional differentiation, antiinflammation, and antifibrosis. Although this approach has been extensively applied in kidney regeneration, this approach is not reviewed in this chapter. Instead, the major role of each bioactive molecule used in kidney regeneration is summarized in Table 45.4.

Other in situ approaches include the recruitment of host stem cells from BM or tissue-specific progenitor cells to injury sites for regeneration or repair [19–21]. A key issue in this in situ tissue regeneration approach is how to efficiently recruit desirable cell types into the damaged tissues. For efficient endogenous stem/progenitor cell mobilizing and recruiting into the site of the injury, chemo-attractants such as granulocyte-colony stimulating factor (G-CSF) and stromal cell–derived factor-1 (SDF-1) have been utilized [19,21,26]. Another issue is developing a scaffolding system to provide microenvironments that the recruited stem/progenitor cells can attach to, differentiate into tissue-specific cell types, and form new tissues. Natural ECM and synthetic polymers have been used as a scaffolding system to promote in situ kidney regeneration as described in "Tissue-engineered cellular three-dimensional renal constructs" section, and stem cell mobilizing or recruiting factors and/or other bioactive molecules capable of promoting cellular functions (Table 45.4) can be incorporated within the scaffolding system. In this section, we will review two representative stem cell mobilizing or recruiting factors, G-CSF and SDF-1 for in situ kidney regeneration.

Granulocyte-colony stimulating factor

In early studies, G-CSF was used clinically to treat neutropenia induced by chemotherapy in cancer patients due to its primary role in stimulating the production of granulocytes [180]. Recent findings on G-CSF's effects on stem cell mobilization [e.g., hematopoietic stem cells (HSCs), MSCs] from BM to peripheral blood have opened a new area of research to regenerate damaged organs [181,182]. Numerous clinical studies of G-CSF for organ regeneration have been reported in patients with myocardial infarction [183], ischemic cardiomyopathy [184], myelopathy [185], acute spinal cord injury [186], and muscular dystrophy [187]. With these promising results, several preclinical studies have been conducted using the administration of exogenous G-CSF to regenerate damaged kidneys [135–137,188–190].

For instance, Iwasaki et al. reported that the administration of exogenous G-CSF into AKI-induced mice successfully mobilized BM-derived cells into the peripheral circulation [136]. These BM-derived cells migrated into damaged renal tubules, resulting in increased survival rates and inhibited deterioration of renal functions—promoting reduced serum creatinine and blood urea nitrogen levels.

TABLE 45.4 Bioactive molecules for in situ kidney regeneration.

Bioactive molecules	Major role in kidney regeneration	Refs.
G-CSF	BM-derived stem cells' mobilization, alteration of inflammatory kinetics, antiapoptotic effects of renal tubular epithelial cells, inhibited deterioration of renal function	[135–138]
SDF-1	BM-derived stem cells' recruitment, protection of renal function, antiapoptosis of renal tubular epithelial cells	[24,139–142]
HGF	Antiapoptotic, mitogenic, motogenic, and morphogenic effects on renal tubular cells and angiogenic and angioprotective effects on endothelial cells, antifibrosis	[143–145]
IGF-I	Enhancement of glomerular filtration, renotropic property on renal tubules, improvement of renal function	[146,147]
EGF	Mitogenic effects of renal tubules, improvement of renal function	[148–150]
VEGF	Antiapoptosis and proliferation of endothelial cells, preservation of renal hemodynamics and function, antifibrosis	[151–153]
TGF-β	(Detrimental) Renal fibrosis	[154,155]
BMP-7	Potent inhibitor of TGF-β1 induced epithelial-to-mesenchymal transition of proximal tubular epithelial cells, differentiation and survival factor for podocytes, antifibrosis, diminishing the activation of tubulointerstitial inflammation and fibrosis, preserving renal function	[156–159]
IL-22	Proregeneratory effect on tubular epithelial cells, ameliorates renal ischemia reperfusion injury	[160,161]
Erythropoietin	Antiapoptosis, renoprotective effects, antiinflammation, antifibrosis	[162,163]
Melatonin	Antioxidant and antiapoptotic effects	[164,165]
Galectin-3	Enhancement of nephrogenesis, (detrimental) interstitial fibrosis and progression	[166,167]
Vitamin E	Antioxidant effect	[168]
Activin A	(Detrimental) Inhibition of branching tubulogenesis, profibrotic factor	[169,170]
Follistatin	An antagonist for activin A, antiapoptosis and proliferation of renal tubular cells	[170]
PDGF	(Detrimental) Potent mitogen for mesangial cells, progression of glomerular nephritis, fibrosis	[171,172]
Angiotensin II	(Detrimental) Renal tubular cells apoptosis	[173]
Rapamycin	Reduced inflammation	[174–176]
Anti-HE4 antibody	Antifibrosis	[177]
HDAC inhibitor	Antifibrosis	[178,179]

BM, Bone marrow; *BMP*, bone morphogenetic protein; *EGF*, epidermal growth factor; *G-CSF*, granulocyte-colony stimulating factor; *HDAC*, histone deacetylase; *HE4*, antihuman epididymis protein 4; *HGF*, hepatocyte growth factor; *IGF*, insulin-like growth factor; *PDGF*, platelet-derived growth factor; *SDF*, stromal cell–derived factor; *TGF*, transforming growth factor; *VEGF*, vascular endothelial growth factor.

Fang et al. also reported similar results using G-CSF of boosted migration of BM-derived cell into the ischemic kidneys that could proliferate and differentiate into renal tubular epithelial cells [137]. In another study the antiapoptotic effect of G-CSF to renal tubular epithelial cells was also demonstrated [135].

Despite the favorable results of G-CSF on treatment of renal injuries, the therapeutic effects are still controversial. Tögel et al. and Stokman et al. reported the detrimental role of granulocytes that comigrated into ischemic kidney [139,188]. Indeed, G-CSF stimulates the BM and activates the mobilization of not only BM-derived cells but also neutrophils and macrophages [138]. Alteration of inflammatory kinetics with the use of G-CSF may result in further injury both in experimental animal models and in human patients [188,191]. Therefore careful administration of G-CSF in a controlled manner is necessary for its application to treat renal injuries.

Stromal cell–derived factor-1

Another attractive chemokine that regulates cell migration is SDF-1. It is well known that the retention and mobilization of stem/progenitor cells is controlled by SDF-1/C-X-C chemokine receptor 4 (CXCR4) axis [192]. CXCR4 is one of the major receptors that regulate trafficking of BM-derived stem cells such as MSCs, HSCs, and endothelial progenitor cells (EPCs). Under normal conditions the SDF-1 concentration in BM remains higher than that in the periphery. Stem/progenitor cells reside in BM. However, when tissues get injured, hypoxia-induced factor 1–induced SDF-1 expression increases in damaged tissues. The reversed SDF-1/CXCR4 axis allows stem/progenitor cells to mobilize from the BM to the peripheral blood and then be recruited to the site of injury [193]. Therefore it is possible that local administration of exogenous SDF-1 within injured tissue can generate a higher concentration gradient than that in the natural healing process, thereby recruiting more stem/progenitor cells to the injured sites than in the normal regeneration process, accelerating the regeneration process [22].

Recent reports have demonstrated the feasibility of local delivery of SDF-1 using an engineered scaffolding system for tissue regeneration. Ko et al. developed a SDF-1-loaded poly(L-lactide)/gelatin scaffolding system for local release of SDF-1 in a controlled manner [24]. Subcutaneously implanted scaffolds efficiently recruited host stem/progenitor cells into the implants and the recruited stem cells were capable of multilineage differentiation. The results implicated that local delivery and implantation of SDF-1-loaded scaffolds can be applied for tissue regeneration by mobilizing and recruiting host endogenous stem/progenitor cells into injured sites.

Using similar methods, the therapeutic potential of the controlled delivery of SDF-1 in an ischemia/reperfusion injury of skeletal muscle was demonstrated [194]. SDF-1-conjugated polyethylene glycol-fibrin scaffolds implanted into damaged muscles enhanced cell recruitment and revascularization of regenerating muscle. In another study, implantation of SDF-1-conjugated scaffolds into muscles also efficiently promoted tissue-specific host cell recruitment [23]. Recently, Sun et al. developed a collagen-binding SDF-1 system for sustained and local release of SDF-1 [195]. Implantation of collagen-binding SDF-1 scaffolds successfully recruited endogenous stem cells to the ischemic hearts and improved cardiac function. Koudy Williams et al. reported interesting results that the local delivery of the SDF-1 showed comparable regeneration capability to cell therapy for treating urinary incontinence [196]. In addition, ADM3100, a CXCR4 antagonist, has been widely investigated in preclinical and clinical studies [197,198]. Several clinical studies have demonstrated that the AMD3100 is an effective agent for HSC mobilization, and AMD3100 in combination with G-CSF mobilized higher numbers of HSCs than G-CSF alone [197]. Preclinical studies have showed that the AMD3100 mobilized EPCs and increased the number of circulating angiogenic cells. Those mobilized EPCs were able to home to the sites of vascular injury and enhanced angiogenesis [199,200]. Therefore it is expected that SDF-1-based in situ tissue regeneration strategies can be alternatives to current cell-based therapy models.

These studies suggest that an SDF-1-based approach may play a protective and regenerative role in regenerating damaged kidneys. There is evidence that the SDF-1/CXCR4 axis is one of the key mediators in the migration and homing of stem/progenitor cells for renal regeneration. Previous studies demonstrated that increased SDF-1 or AMD3100 concentration and CXCR4 expression in cells in an ischemia–reperfusion-induced kidney injury model provided pathological and functional protection and prevented renal tubular epithelial cell apoptosis [139–141]. AMD3100 treatments mobilized HSCs and EPCs and reduced levels of blood urea nitrogen and serum creatinine in following kidney ischemia/reperfusion injury in a rat, implying the therapeutic potential for protection after renal failure [142].

Our group has also investigated the renoprotective and regenerative effects of SDF-1 in a rat acute renal failure model. We previously demonstrated that the intrarenal injection of collagen hydrogel can efficiently recruit host renal stem/progenitor cells and MSCs to facilitate the regeneration of glomeruli and tubules and promote functional recovery in ischemia/reperfusion injury–induced kidneys [25]. With this encouraging result, we developed a controlled local SDF-1 delivery system in collagen hydrogel. The implantation of this collagen-based SDF-1 delivery system into kidneys could achieve successful recruitment of renal progenitor cells and MSCs, and restoration of renal function, showing the potential use of this system for future treatment.

Conclusion and future perspectives

Recent progress in tissue engineering and regenerative medicine has demonstrated great potential for regenerating and restoring normal kidney functions in preclinical renal failure models as well as in a number of clinical trials using autologous cells. Recent tissue engineering approaches of cell therapy, engineered 3D renal tissue constructs, decellularization/recellularization strategy, and in situ renal regeneration have achieved successful outcomes in terms of structural and functional improvements following renal damage. However, several challenges still need to be addressed. Development of reliable cell sources, defined culture methods, and expansion techniques is prerequisite.

Advanced 3D renal construct fabrication strategies is needed to mimic structural and functional complexity of the native kidney. There are still uncertain complicated mechanisms of the renal regeneration process. More sophisticated approaches for engineering renal construct implantations are required to address immunological issues, vascularization, innervation, and renal physiology. The only definitive treatment option for renal failure in AKI, CKD, and ESRD is kidney transplantation. However, the shortage of donor kidneys and complications of immune rejections leading to transplant failure present remaining challenges. Whole kidney engineering using the decellularization/recellularization strategy can be an attractive alternative even though long-term patency of the implants has not been demonstrated in vivo. Even though the kidney tissue engineering faces many challenges to be addressed for clinical translation, multidisciplinary research and close collaboration will be able to accelerate generating functional and transplantable kidney for the treatment of kidney diseases.

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Chapter 46

Tissue engineering: bladder and urethra

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Introduction

The entire urinary tract is divided into two parts: the upper (kidney and ureter) and lower urinary tract (bladder and urethra). The function of the urinary system is to remove waste products accumulated from the body, regulate water, and salt balance, and to store and transport urine out of the body. Histologically, the urinary tract consists of urothelium lined lumen surrounded by a collagen-rich connective tissue and smooth muscle layers. In the lower urinary tract, the main source of force that allows for the release of urine is the detrusor muscle, which is a layer of the urinary bladder wall consisting of smooth muscle fibers that are arranged in a spiral, longitudinal, and circular bundles. The smooth muscle tissues can be extended to allow for the storage of urine and contracted to empty the bladder. The urothelium serves as a passive barrier to prevent the absorption of urine and does not generate mucus. This internal lining of the bladder and part of urethral lumen, composed of urothelial tissue and lamina propria, is believed to regulate some aspects of overall bladder physiology in response to stimuli, such as distension during filling [1].

The urinary bladder is one of the most elastic organs in the body and can increase its volume significantly to accommodate up to 800 mL of urine at maximum capacity in adults. Transitional epithelium, elastic fibers, and visceral muscle tissue in the wall of the urinary bladder contribute to its distensibility and elasticity, allowing it to easily extend and return to its original size several times each day. The bladder is connected with the male urethra, which is a narrow fibromuscular tube that passes urine and semen from the bladder and ejaculatory ducts, respectively, to the exterior of the body.

Congenital disorders, cancer, trauma, inflammation, or other conditions of the bladder and urethra can damage the anatomical tissue structure or cause complete loss of function. Both situations usually necessitate eventual reconstruction or replacement of the damaged tissue. Although patients suffering from diseased or injured organs can be treated with transplanted organs, there is a severe shortage of donor organs, which is worsening yearly as the population ages and new cases of organ failure increase. Lower urinary tract reconstruction has been performed with autogenic nonurologic tissues such as gastrointestinal segments, mucosa from multiple body sites, homologous tissues from a donor (cadaver or living donor kidney), or heterologous tissues or substances (e.g., bovine collagen) [2]. All these materials often cause a series of complications after surgery due to the implanted tissue rejection or because of the inherently different functional parameters that cause a mismatch in the system. For example, the replacement of bladder tissue with gastrointestinal segments can be problematic because the normal urothelium excretes proteins [3], whereas gastrointestinal tissue generally absorbs them. This mismatched state can lead to severe metabolic complications in addition to infection and other issues [4]. Therefore replacement of lost or deficient urologic tissues with functionally equivalent ones would certainly improve the outcome of reconstructive surgery in the genitourinary system. This goal may soon be achievable with the use of tissue engineering techniques.

Tissue regeneration uses the principles of cell biology, biomaterials science, bioactive factors, and biomedical engineering to develop biologic substitutes that can restore and maintain the normal function of damaged or lost tissues and organs. Tissue engineering involves the use of natural or synthetic matrices, often termed scaffolds, which encourage the body's natural ability to repair itself and assist in determining the orientation and direction of new tissue growth. Often, tissue engineering uses a combination of these techniques. For example, matrices seeded with cells can be implanted into the body to encourage the growth or regeneration of functional tissue.

In the last two decades, scientists have attempted to cultivate native and stem cells, engineer tissues and

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develop treatment modalities using regenerative medicine techniques for the urinary tract system. This article reviews the progress of the bladder reconstruction efforts with tissue engineering technology.

Cell sources

Bladder and ureter cells

Although implanted tissue can be heterologous, allogeneic, or autologous in experimental animal models, only the autologous cells are considered as a viable source for clinical use currently [5] to avoid graft rejection and long-term use of immunosuppressive medications after allogeneic transplantation. Autologous somatic cells obtained from the bladder or ureters [6] are often used for tissue-engineered bladders. The donor tissue is dissociated into individual urothelial cell (UC) and smooth muscle cell (SMC), which are expanded in culture, attached to a support matrix, and then implanted back into the host. Ideally, this approach allows for the augmentation of the bladder tissue function [7–10]. However, suitable bladder cells from the patient for this purpose are sometimes limited or unobtainable because of the disease conditions, such as malignancy.

Stem cell sources

Adult stem cells could be a suitable alternative to the bladder cells. They are an appealing cell source for tissue regeneration due, in part, to their self-renewal, long-term expansion in vitro, and differentiation potential. Three types of cell sources have been used for bladder regeneration in experiment models that are autologous cells: UCs and SMCs, urothelial progenitor cells [11,12], bone marrow stromal cells (BMSCs) [13,14], adipose stem cells [15,16], urine-derived stem cells (USCs) [17–20], and hair follicle stem cells [21,22]; allogeneic cells: UC + SMC [23] and mesenchymal stem cells (MSCs); and xenogenous cells: amniotic fluid–derived cells [24], dental pulp stem cells [25], hBMSCs + HSPCs (hematopoietic stem/progenitor cell) [14], and genetically modified hBMSCs [26]. Different types of stem cells used for bladder reconstruction are listed in Table 46.1.

Adult stem cells avoid some ethical issues associated with embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). They do not transdifferentiate into a malignant phenotype; thus there is a diminished risk for teratoma formation or immunogenicity after the cells are implanted in vivo. In addition, these cells possess paracrine effects, immunomodulatory properties, are immune privileged (i.e., tolerate the introduction of antigens without eliciting an inflammatory immune response), and have a low immunogenic potential [28]. Adult stem cells, especially MSC and hematopoietic stem cell, are well-investigated cell types in stem cell biology [29]. Adult stem cell research remains an area of intense study, as their potential for therapy may be applicable to various degenerative disorders. Many other types of adult stem cells have been identified in organs throughout the body and are thought to serve as the primary repair entities for their corresponding organs. The discovery of such tissue-specific progenitors has opened up new avenues for research. Within the past decade, adult stem cell populations have been found in many adult tissues other than the bone marrow, skin [30], and adipose tissue [31], including those traditionally considered a postmitotic organ, such as brain [32], heart [33,34], and urine [35].

ESCs or iPSCs exhibit two remarkable properties: the ability to proliferate in an undifferentiated but still pluripotent state (self-renewal) and the ability to differentiate into different specialized cell types [36]. These cells have been maintained in the undifferentiated state for at least 80 passages and 200 population doublings (PDs) in vitro when grown using currently published protocols [37,38]. In addition, human ESCs differentiate into cells from all three embryonic germ layers in vitro. However, these cells are not currently used for many clinical applications because of the risks for immunogenicity and teratoma formation, in addition to the ethical dilemmas associated with the manipulation of embryos in culture.

Although the entire urinary tract system is responsible for excreting ions, metabolic products, and toxic wastes from the body, it is a closed system with a germ-free environment—unlike the digestive system, which is open and does not have sterile conditions. There are some living cells in urine that can survive for a few hours within the sterile urinary system. Stem cells exist in human voided urine and urine drained from the upper urinary tract. These cells, termed USCs [35], possess stem cell characteristics with clonogenicity, cell growth pattern, robust proliferative potential, and multipotential differentiation. These cells can be obtained using simple, safe, noninvasive, and low-cost procedures, thus avoiding the adverse events associated with obtaining cells from tissue sources. A single USC can expand to a large population with 60–70 PDs [39,40]. When seeded onto a scaffold and subcutaneously implanted into nude mice, multilayered tissue-like structures are formed consisting of urothelium and smooth muscle. In addition, USCs differentiated into endothelial, osteogenic, chondrogenic, adipogenic, skeletal myogenic, and neurogenic lineages. However, implantation of these cells in vivo, observed for up to 3 months, did not form teratomas despite the telomerase activity (TA). USCs have been used in cell-based therapies and urogenital tissue engineering applications, including urethral tissue repair [40,41], bladder reconstruction [42], kidney and urethral sphincter [43,44], vesicoureteral reflux [43], and erectile dysfunction [45,46].

TABLE 46.1 Stem cell properties for bladder regeneration.

	MSCs	Primitive stem cells	USCs	ESCs/iPSCs	Somatic cells
Cell types	• BMSCs [13,14] and ASCs [15,16] • Urothelial progenitor cells [11,12] • Hair follicle stem cells [21,22] • Dental pulp stem cells [25] and hBMSCs + HSPCs [14] • Genetically modified hBMSCs [26]	AFCs [24,27] or placenta-derived stem cells	USCs [17–20]		UC + SMC [23]
Self-renewal and expand capability	Limited, PD ~ 30	High, PD	High, PD 60–70	Very high, PD > 200	Limited, PD < 30
Multilineage differentiation capability	Multipotent, but mainly limited within mesodermal cell lineages	Yes	Multipotent differentiation potential	Pluripotent (can form all lineages)	Non
Urothelial, endothelial, or myogenic differentiation capability	Very low	High	High	Low	Non
TA/telomere length	Cannot be detected	Yes	Up to 75% USC clones possess TA and relative long telomere	Possess TA and long telomere	Non
Harvest approach	Invasive, relatively hard to harvest bone marrow stromal cells at the senior	Aspirate from amniotic fluid	Noninvasive, simple, cost-low, safe	Invasive for iPSCs	Invasive
Pure stem cell isolation	Hard isolate pure stem cell	Easy	Very easy	Easy	Non
Number of stem cells harvested	1 MSC/10^4 bone marrow stromal cells at new bore baby, 1 MSC/10^6		100–140 USC clones/24 h urine in adult		Non
Rejection after implanted in vivo	Autogenous or allogeneic	Allogeneic	No rejection	Likely to be rejected if donor is unmatched	No rejection if autogenous cells are used
Oncogenic potential	No reported	No reported	No reported	Yes, harbors disease-causing genes of donor	No reported

AFCs, Amniotic fluid–derived cells; *ASCs*, adipose stem cells; *ESCs*, embryonic stem cells; *iPSCs*, induced pluripotent stem cells; *MSCs*, mesenchymal stem cells; *PD*, population doubling; *SMC*, smooth muscle cell; *TA*, telomerase activity; *USCs*, urine-derived stem cells; *UCs*, urothelial cells.

In chronic bladder diseases, USCs might be a suitable cell source for bladder tissue regeneration because cells from the upper urinary tract are normal. In the treatment of end-stage bladder diseases or muscle-invasive bladder cancer, using engineered bladder tissue with USCs would be superior to bladder reconstruction using intestinal segments. Intestinal segments used for bladder reconstruction appear to be at an increased risk for malignancy, particularly adenocarcinoma, because of histologic changes in the intestinal mucosa after long-term exposure to urine. Recent studies showed that all children with neurogenic

bladder disease are at increased risk of bladder cancer regardless of exposure to intestine [47]. Bowel tissue also increases the risk of complications such as stone formation and excess mucous secretion [48]. Harvesting USCs from patients who already have a nephrostomy tube in place would be a simple, noninvasive, and low-cost approach to obtaining cells for engineering bladder tissue.

Other sources of stem cells include the amniotic fluid and placenta. These contain multiple partially differentiated cell types derived from the developing fetus [27,49]. Stem cell populations have been isolated from these sources and express ESC and adult stem cell markers, but they do not form teratomas. The cells are multipotent and can differentiate into cells from all three germ layers. In addition, such cells have high replicative potential and could be stored for future self-use, without the risks for rejection and ethical concerns.

Mechanism of cell therapy

Cell expansion

Fetal stem cells, such as amniotic fluid and primitive stem cells (such as placenta-derived stem cells), showed promise for future clinical applications [27,49]. These cells can give rise to cells from the endoderm, mesoderm, and ectoderm and can be maintained for over 250 PD. Long telomeres are retained and a normal karyotype without tumorigenicity is observed in vivo [50]. Adult stem cells have been successfully isolated from various types of tissues. These cells usually reach a PD rate of 20−40 in 10 passages [19].

USCs can generate large numbers of cells in a single clone [20,40]. Around 100−140 USC clones can be formed from 24-hour urine collection from one individual [51]. Up to 75% of these cells are highly proliferative due to their relatively higher TA (USC-TA$^+$) and longer telomeres compared to bone marrow stem cells (BMSCs) [20]. USCs are reported to have a PD rate of 60−70 for up to 20 passages, whereas other USCs without TA can be maintained for 8−10 passages with 34 PDs. Based on this ratio of cells and urine volume, two urine samples containing 20−30 USC clones could potentially yield at least 1.5×10^9 USCs at the end of passage 4 within 4−5 weeks [5,39].

Isolation of USCs is a separation- and digestion-free procedure. Urine samples are simply centrifuged, and cells are seeded in a mixed media composed of keratinocyte serum-free medium and embryonic fibroblast medium in a 1:1 ratio [35]. Expanded USCs are a relatively homogenous population and only require 2%−5% serum to be maintained in vitro; by contrast, most MSCs require 10%−20% serum [20]. When cells collected from voided urine are cultured in USC culture media, only USCs tend to attach to the culture container and continuously expand in culture [20]. This quick, easy, and economical process for USC isolation may also facilitate their large-scale expansion for potential clinical trials.

Multipotentiality

During tissue repair, stem cells accelerate tissue repair and regeneration in various ways (Table 46.2). Although ESCs and iPSCs are pluripotent and can differentiate into multiple specialized cell types [36], their differentiation capability into urologic-specific cell types is relatively low [19]. Under appropriate conditions, BMSCs can be successfully induced into cells with bladder SMC characteristics, both in vitro and in multiple animal models [57−59]. Induced BMSCs proliferate at a similar rate as bladder SMCs and possess a similar histologic appearance and contractile phenotype. However, only 5%−10% of cells in BMSCs can be induced into urothelial-like cells

TABLE 46.2 Multiple modes of action assigned to adult stem cells.

Multiple functions of stem cells	Outcomes and potential applications	References
Multipotent differentiation	Osteocytes, chondrocytes, adipocytes, myocytes, epithelial, and endothelial cells for cell replacement	[20,39]
Secretion of trophic factors	Recruitment resident cells to tissue repair	Liu et al. [52]
Secretion of extracellular matrix	Prompt cell proliferation, rejuvenation, and differentiation of bone marrow-derived stem cells	[53]
Immunomodulatory and antiinflammatory	Inhibited T- and B-cell proliferation to decrease fibrosis	[54]
Gene delivery via angiogenic growth factor gene transfection	Soluble factor gene therapy to accelerate tissue regeneration at the site of chronic injury with extensive scarring	[45,55,56]

with specific lineage marker expression [60–65]. One reason for this could be their relative scarcity; only an estimated 1 in 1 to 1.5×10^4 cells in the bone marrow is BMSC [66].

Although the effects of aging on MSCs remain controversial, some studies show an age-related decline in stromal vascular fraction number, proliferation rate, longevity, differentiation potential, and MSC immunophenotype expression in BMSCs from older compared to younger donors [67–71]. This question becomes of great importance, given that the aging population is increasing, and thus the prevalence of urologic diseases also is increasing. Considering the large quantity of MSCs (up to 10^9) required for clinical applications, especially for bladder reconstruction, it is critical to find a stable cell source with potent MSCs [5].

Importantly, unlike many other cell lineages, USCs can efficiently differentiate into urologic tissue-specific cell lineages, including UCs, SMCs, and Endothelial cells (ECs). They have higher differentiation frequency in UCs than BMSCs. Under the same induction conditions for 7 days, up to 60%–70% USCs are induced into a uroepithelial lineage, compared to only 5% for BMSCs [20,58]. If induction extended to 14 days, up to 90% of USCs are differentiated. Cells develop a cobblestone-like morphology and express both urothelial-specific cell markers (uroplakin-III and uroplakin-Ia) and generic epithelial cell markers (CK7 and AE1/AE3). Differentiated USCs also present tight junction markers (ZO-1, E-cadherin, and cingulin) in a dose-dependent and time-dependent manner [20]. Those cells display enhanced barrier function with at least a 60% decrease in leakage than noninduced cells [20].

USCs-derived UCs were seeded on a porcine small intestinal submucosal scaffold and cultured in vitro for 14 days before implantation into nude mice. Constructs generated stratified layers in vivo, and neo-tissue expressed urothelial-specific cell markers (uroplakin-III and uroplakin-Ia). USCs have comparable differentiation potency into SMCs as BMSCs [60]. Up to 80% of induced USCs express early smooth muscle differentiation markers (α-smooth muscle actin and calponin), contractile SMC markers (desmin and myosin), and smooth muscle-specific marker (smoothelin) after 14 days induction with smooth muscle differentiation media in vitro [20]. Cells exhibit rapid lattice contraction as mature SMCs. When implanted in vivo on a porcine small intestine submucosal scaffold, those cells formed multiple layers of SMCs beneath the UC layers and presented SMC markers (desmin, myosin, α-smooth muscle actin). USCs are also capable of endothelial differentiation with a barrier function. EC-induced USCs display vessel-like structures on a solidified Matrigel surface after in vitro induction. Those cells express EC-specific genes (vWF and CD31), proteins (CD31, vWF, KDR, FLT1, and eNOS), and tight endothelial junction marker (VE-cadherin). When implanted subcutaneously into athymic mice, EC-induced USCs effectively form neo-vessel structures [72].

Paracrine effects and immunomodulatory properties

Stem cells have two important roles in tissue regeneration. First, they can replace diseased cells by engrafting, cell fusion, and differentiating into the required host cell type (e.g., bone marrow transplant and cell therapy for myocardial ischemia). Second, grafted cells stimulate the host's cells to repair the injured tissue, without the donor cells contributing directly to form the new tissue. This occurs because the grafted cells secrete factors that signal the host's cells to change their biological behavior and the microenvironment. The signaling from one cell to another is referred to as paracrine effect.

Adult stem cells can invoke a paracrine response [73]. The implanted stem cells secrete factors that promote angiogenesis and protect against apoptosis, fibrosis, and inflammation [13,74,75], which prompt the host's cells to repair the tissue themselves. Humans and most mammals have a wound repair mechanism that, on its own, can only be able to repair small wounds, but not for large wounds. Large wound is often remodeled by scar tissue. However, human tissue still possesses regenerative potential once it receives the appropriate signals to initiate internal tissue regeneration and repair provided by paracrine effects of the grafted stem cells.

Although stem cells are known to have a short life span (1–3 weeks) after implantation, they have long-term effects on tissue repair. For example, they appear essential for initiating tissue regeneration but are expendable once the patient's cells are activated. Paracrine effects are amplified once the grafted cells are attracted to injured tissues. Cells within the damaged tissues often secrete cytokines, regulatory factors that act as mediators to generate an immune response that attracts grafted cells. Sequentially, the grafted cells secrete their own cocktail of proteins that stimulate the host's stem cells and inhibit inflammation and oxidative stress, protect against fibrosis, promote resident cell proliferation, and increase vascularization and blood flow into the injured areas.

In addition, the paracrine effects of adult stem cells can reduce immune response and possess immunoregulatory properties. For example, regulatory T cells play an important role in the induction of peripheral tolerance, inhibition of proinflammatory immune responses, and decreased immune reactions. To test the immunomodulatory effects of USCs, our studies demonstrated that USCs can inhibit proliferation of peripheral blood mononuclear cells (PBMNCs) (T and B cells), and secreting interleukin

(IL)-6 and IL-8 [76]. In the specific experiment the mononuclear cells usually proliferate when mixed with other somatic cells due to immune stimulation. In contrast, PBMNC concentrations in USC wells are much lower than in somatic cells, even BMSC culture wells [76]. Colorimetric enzyme-linked immunosorbent assay (ELISA) showed less BrdU labeled in the USC−PBMNC mixed culture wells and more in BMSC culture wells. As costimulatory molecules, CD80 and CD86 initiate and modulate the T-cell immune response. Both CD80 and CD86 expressed on the surface of antigen-presenting cells interact with cytotoxic T-lymphocyte antigen-4 expressed on activated T cells and mediate critical T-cell inhibitory signals. About 3% of the BMSCs were positive for CD80 (vs 1.05% of USCs), and 1.3% of the BMSCs were positive for CD86 (vs 0.55% of USCs). Human cytokine release arrays showed that IL-6 and IL-8 concentrations were elevated after stimulation by PBMNCs in USC supernatant, higher than BMSC supernatant. Thus it indicates that IL-6 and IL-8 might be the main immunomodulatory cytokines to target in future studies aimed at preventing and treating bladder abnormalities that accompany diabetes, other immune system disorders, or rejection of transplanted organs.

Biodegradable biomaterials

A biological scaffold tailored to stem cells is central to mimic the function of the extracellular matrix (ECM) in tissue engineering. The ECM provides structural support and a physical environment so that cells can attach, proliferate, migrate, differentiate, and function [77]. It confers mechanical properties to tissues and delivers bioactive cues for regulating activities of residing cells and provides a dynamic environment for vascularization and new tissue formation. Scaffolds can be designed to stimulate and direct tissue formation to replace portions of tissues or whole tissue structures. The material would possess appropriate porosity and interconnectivity between the pores to facilitate cell attachment, migration, penetration, differentiation, tissue growth, and integration. The ideal type of cell replacement should be composed of materials with similar physical and mechanical properties as the native tissue and should degrade at the same rate as the new tissue is generated. Porosity should allow nutrient transfer and cell adhesion without compromising mechanical strength. Two categories of scaffolds designed to carry cells include synthetic scaffolds and natural collagen matrix for the bladder regeneration (Table 46.3).

Synthetic scaffolds

Various porous structures composed of natural or synthetic biodegradable and biocompatible materials have been used as scaffold carriers. Some biomaterials have been approved by the US Food and Drug Administration (FDA) for the use in medical devices in humans. These include polyesters of naturally occurring a-hydroxy acids such as polyglycolic acid (PGA), polylactic acid, poly (lactic-*co*-glycolic acid), and polycaprolactone. Metabolites formed after degradation of these materials have been confirmed as nontoxic and are eventually eliminated from the body as carbon dioxide and water [91]. Because these polymers are thermoplastic, they can easily be fabricated into a three-dimensional (3D) scaffold with the desired microstructure, gross shape, and dimensions by various techniques, including molding, extrusion [92], solvent casting [93], phase separation, and gas foaming techniques [94]. In addition, synthetic polymers can be manufactured on a large scale using various techniques, including electrospinning [95], phase separation, gas foaming, particulate leaching, inkjet printing, and chemical cross-linking. The strength, degradation rate, and microstructure may be adjusted during manufacturing. Scaffolds can be made in different shapes and porosity to facilitate cell engraftment, or further modified by incorporation, surface adsorption, or chemical attachment of bioactive factors. However, one drawback of synthetic polymers is the lack of biologic recognition. To incorporate cell recognition domains into these materials, copolymers with amino acids have been synthesized [96]. Other biodegradable synthetic polymers, including poly(anhydrides) and poly(ortho esters), can also be used to fabricate scaffolds with controlled properties [97].

Biodegradable properties

Biodegradable biomaterials in tissues of the urinary tract system function as an artificial ECM to replace the biologic and mechanical functions of native ECM. These biomaterials facilitate localization and delivery of cells or bioactive factors (e.g., cell-adhesion peptides and growth factors) to desired sites in the body and define a 3D space for the formation of new tissues with appropriate structures. They also serve as a guide for the development of new tissues with the appropriate function. Direct injection of cell suspensions without such matrices has been used in some cases [98]. However, without this scaffold function, the localization of transplanted cells is difficult to control.

For cell-based tissue engineering, expanded cells are seeded onto a scaffold synthesized with the appropriate material. Since most mammalian cell types are anchorage dependent and will die if no cell-adhesion substrate is available, biomaterials provide such a substrate capable of delivering cells to specific sites with high loading efficiency. Biomaterials can also provide mechanical support against in vivo forces, maintaining the predefined 3D

TABLE 46.3 Summary of advantages and limitations of biodegradable materials for bladder regeneration.

	Advantages	Limitations
Synthetic scaffolds		
• PGA/PLGA • PLLA • Collagen–fibrin hybrid scaffold with IGF1 [78] • Acellular collagen-heparin scaffolds with growth factors [79] • VEGF-loaded nanoparticle-modified BAMAs [80] • PGA with PGA/chitosan "sandwich" graft [81]	• Renewable, sustainable, and biodegradable • Safe for use, nontoxic, free-immunogenic, free-carcinogenic, and free-thrombogenic • Ease of manufacturability • Reasonable cost • Durable and storage stability • Process ability: fine complex architectures such as porosity rate, pore size can be generated; highly controllable • Consistent products	• Take time to be degraded • Poor biocompatibility • Potential toxic degradation of by-products • Lacks specific integrin-binding site
Biological scaffolds		
SF [23,82,83] or BAMG–SF matrix [83]	• Unlimited source biomaterials • Easy to produce • Most allogenic or xenogeneic antigen contents can be removed after decellularization • Native architecture is preserved • Growth factors and cytokines bound with matrix remain • Integrin-binding sites are retained • Easy to suture to bladder tissue	• Variability depending on the source of biomaterial • Antigenicity • Weak biomechanical properties • Not suitable for subtotal cytoplasty • Take time to produce in the lab
SIS [84–86] and BSM		
BAMG [15,24,87] or UBM [88]		
Decellularized colon matrix [89]		
Matrix with hydrogel (such as HA–PLGA-modified SIS [90])		

BSM, Bladder submucosa matrix; *BAMA–SF*, bladder acellular matrix graft–silk fibroin; *BAMG*, bladder acellular matrix grafts; *HA*, hydroxyapatite; *IGF1*, insulin-like growth factor 1; *PGA/PLGA*, polyglycolic acid/polylactic-*co*-glycolic acid; *PLLA*, poly-L-lactic acid; *SIS*, small intestine submucosa; *UBM*, urinary bladder matrix; *VEGF*, vascular endothelial growth factor.

structure during tissue development. Furthermore, bioactive signals, such as cell-adhesion peptides and growth factors, can be loaded to help regulate cellular function. Generally, two classes of biomaterials are used for engineering of genitourinary tissues: synthetic polymers and tissue-derived matrices. Although synthetic polymers can be produced on a large scale with controlled properties of strength, degradation rate, and microstructure, naturally derived materials and acellular tissue matrices have the potential advantage of biologic recognition, which can lessen host-versus-graft reactions.

Limitations of nutrient and gas exchange currently restrict tissue-engineered implants to a volume of approximately 3 mm^3. Therefore vascularization of the regenerating tissue is essential in order to engineer large complex tissues and organs. Three approaches have been used to encourage the vascularization of bioengineered tissue. First, incorporating angiogenic factors into bioengineered tissue can attract host capillaries and enhance neovascularization. Second, some studies have investigated the effects of seeding endothelial cells with other cell types in the bioengineered tissue [75]. Finally, vascularizing the matrix before cell seeding has been attempted. There are many obstacles to overcome before large tissue-engineered solid organs are produced; however, recent developments may provide important knowledge and essential materials to accomplish this goal.

Porosity

Recellularization of biological materials (scaffold) with cell-seeded technology provides a promising option in tissue regeneration. The scaffold needs to have a 3D structure with high porosity but also maintains a normal tensile strength. A 3D scaffold with higher porosity and relatively larger pore size (50–200 μm) promotes cell proliferation, migration, and infiltration into the matrix and appears to allow abundant cell loading onto the scaffold, thereby promoting in vivo tissue regeneration [99–101]. Treatment with 5% peracetic acid solution (PAA) led to high porosity on the surface of the matrix with less cellular material retained, and about 75% of normal tensile strength remained. Cells penetrated deeper into the lamina propria of the matrix compared to untreated matrix [102].

Natural collagen matrix

Naturally occurring matrix materials may also function as 3D scaffolds in tissue engineering and regenerative

medicine, including both decellularized natural matrix and matrix produced from naturally extracted polymers. Natural materials are widely used in tissue regeneration such as bladder submucosa matrix (BSM) and small intestinal submucosa (SIS); and naturally derived materials, such as collagen, alginate, and silk [23,82,103]. As the most abundant structural protein in the body, collagen is an FDA-approved biocompatible material for various medical uses. Natural decellularized ECM retains tissue-specific architecture and provides various biological and physical material properties specified by the nature of the original tissue. Such matrix materials also share highly conserved matrix proteins among species, such as collagen, laminin, and fibronectins, suggesting they could be nonimmunogenic and attractive for recellularization and tissue integration.

Under certain circumstances, UCs and SMCs differentiated from adult stem cells can form multiple uniform layers on porcine decellularized SIS scaffold in vitro and in vivo, showing the potential of this 3D cell/matrix to develop into a multilayer mucosal structure similar to native urinary tract tissue [41]. When seeded with USCs, bacterial cellulose scaffolds represent a promising material for urinary conduits with a multilayered urothelium and cell/matrix infiltration in vitro and in vivo [40]. However, in the context of future clinical applications, the decellularized matrix could be contaminated by xenogeneic factors. There is also a risk of incomplete decellularization and residual cell bodies, and altered tissue properties due to complete decellularization and deproteinization [104,105].

Acellular tissue matrices

Acellular tissue matrices are collagen-rich matrices prepared by removing cellular components from tissues, often by mechanical and chemical manipulation of a segment of bladder tissue and small intestine [41,106]. Matrices slowly degrade after implantation and are replaced and remodeled by ECM proteins synthesized and secreted by transplanted or ingrowing cells. Acellular tissue matrices support cell ingrowth and regeneration of genitourinary tissues, including urethra and bladder, with no evidence of immunogenic rejection [106]. Because protein structures (e.g., collagen and elastin) in acellular matrices are well conserved and normally arranged, the mechanical properties of acellular matrices are not significantly different than those of native bladder submucosa [107].

Collagen

Collagen is the most abundant and ubiquitous structural protein in the body and is readily purified from both animal and human tissues through enzyme treatment and salt/acid extraction. Collagen exhibits minimal inflammatory and antigenic responses and is approved by the FDA for many types of medical applications, including wound dressings and artificial skin [108]. This material can be processed into various structures such as sponges, fibers, and films [109].

Silk

Silk fibroin (SF) is a protein obtained from *Bombyx mori* cocoons and may have potential uses in low urinary tract reconstruction. SF contains up to 90% of the amino acids glycine, alanine, and serine, compared to other natural biomaterials. SF has excellent biocompatibility and low inflammatory properties and can be fully degraded by naturally occurring proteolytic enzymes [110]. SF has used as an effective biomaterial for bones [111], cartilages [112], blood vessels [113], peripheral nerves [114], cornea [115], bladder [116], and urethra [117], among other tissues. Silk processing methods allow construction of films [118], foams [119], hydrogels [120], gel−spun matrices [116], and woven or nonwoven meshes [121]. Electrospinning may also be an appropriate technique for urethral reconstruction with SF, because it can create a 3D and highly porous scaffold in a conformation that mimics ECM structure in vivo [95].

Matrix binding with growth factors

Tissue engineering approaches should mimic the in vivo setting by providing a biocompatible scaffold, a cell source of appropriate progeny or type, nutrients and other environmental conditions, and appropriate bioactive factors. Localized delivery of bioactive factors is considered to be effective and necessary in mimicking the natural microenvironment of cells within specific tissues. Delivering biomolecular cues, especially growth factors in solution, is difficult because of their rapid diffusion to extracellular fluids, and growth factors always have a limited half-life in vivo. However, sustained delivery of growth factors is important for clinical applications of tissue engineering.

Vascular endothelial growth factor (VEGF) is an important active protein for inducing angiogenesis in tissue engineering, but it requires a delivery system targeted to a given site. There are several ways that the matrix could bind with growth factors. Native human VEGF fused with a collagen-binding domain (CBD-VEGF) can bind to collagen specifically to exert angiogenesis effects that promote regeneration. CBD-VEGF was constructed by linking a sequence that encodes the CBD (TKKTLRT) with VEGF complementary DNA. CBD-VEGF was inserted into one plasmid (pET-28a). The plasmid was transformed into the BL21 strain of *Escherichia coli*. CBD-VEGF could be purified from the protein made from *E. coli*. When collagen was used as a scaffold in

TABLE 46.4 **Experimental animal models for the lower urinary tract reconstruction.**

Surgical procedures	Advantages	Challenges
For the bladder		
Hemicystoplasty	• Evaluation of cell-seeded or nonseeded tissue-engineered technology for the bladder augmentation • The neobladder often retain contractile function because of original bladder + regenerated bladder • histologically, fully regenerated urethelium; partial muscle regeneration and innervation	Stone formation, graft shrinkage
Subtotal cystoplasty	• Assessment of cell-seeded tissue engineering technology for the bladder replacement • Acts as the urinary reservoir • Regenerated urothelium • Bladder capacity increases at the early stages	• High risk of graft collapse or shrinkage and bladder stone formation • Limited innervation, myogenesis, and blood supply at center of the graft • Bladder volume may decrease over time
For the urethra		
Full circumferential repair	• Replacing urethra with severe urethra scarring • Commonly used in preclinical studies • Cell-seeded scaffold is required	Successful rate urethroplasty of relatively low due to heavy scar tissue, poor blood supply, chronic information in the wounded urethra tissue
Inlay or onlay repair	• Used for most cases with urethra restructures • Commonly used in clinic for the patients • Cell-free scaffold could achieve good outcome if the urethral segment is <3 cm in human	Potential infection risk in the local urethral tissue
Animal models		
Large animals		
Canine [24,87,90,123]; porcine, i.e. juvenile Yorkshire swine [28,82]; rabbit [23]; sheep [79]; nonhuman primates [13]	• Physiology, anatomy, and histology similar to humans • Autograft models • Easy development of subtotal cystoplasty model • Test both synthetic or thicker nature-based collagen scaffold with cells • Easy to perform urodynamic studies • Enough tissue regenerated to do contractility studies of muscle strips in organ bath	• High cost to purchase and maintain large animals • Not easy to fix a urethral catheter after surgery in pigs and nonhuman primates

(*Continued*)

TABLE 46.4 (Continued)

Surgical procedures	Advantages	Challenges
Small animals		
Athymic rodent [26] and rat [15,78,83,89]	• Easy to maintain • Well-known histology • Allogeneic and xenogeneic-grafted stem cells not rejected by the host • Economical • Commonly used for hemicystoplasty • Dynamically observe bladder regenerative processes at the different time points • Allogeneic or even xenograft models	• Subtotal cystoplasty model not possible • Require thinner scaffold • High risk of stone formation • Requires special fine devices for urodynamic study and images • Cannot observe immune reaction within graft in immunodeficient rodents

tissue engineering that CBD-VEGF can bind to in vitro and in vivo, VEGF could maintain activity [122].

Preclinical models

Tissue regeneration models

Two in vivo experimental models have been frequently used for bladder regeneration, including hemicystoplasty (in which 40%−50% bladder tissue is removed) and subtotal cystoplasty (in which >75% bladder tissue is removed or trigone-sparing cystectomy) (Table 46.4). Cell seeded or cell free−seeded scaffolds are capable of increasing bladder volume in small animals after hemicystectomy, while cell-seeded scaffolds are often designed for the replacement of diseased bladder in a larger animal model after subtotal cystectomy. A rodent model such as rat is often used to test new cell sources or new biomaterials and study histologic changes in the regenerative bladder. Larger animal models are used to evaluate the effectiveness of bladder function and volume, and anatomical and histological structure changes (Table 46.5). Beagles and minipigs have been used widely used as larger animal models for preclinical studies.

For urethra applications, two surgical approaches have been frequently used for urethral reconstruction: full circumferential and inlay procedures (Table 46.4). The full circumferential technique is used in the cases with significant urethral scarring or lichen sclerosis, while the onlay or inlay procedures have been applied for partial replacements. Depending on the patient and local factors, procedures can be performed as a one-stage procedure or as a planned multistage procedure. More challenges exist in the full circumferential repair for urethra reconstruction due to the large size of graft used, limited blood supply, and free-infected wound in the urethra. For urethra reconstruction studies, the most commonly used animal models are medium or large animals such as rabbit [124] and dog [125]. In either the full circumferential or inlay repair, the cells-seeded scaffolds significantly reduced the probability of side effects in the experimental animals, compared to biomaterial alone used [126]. The majority of preclinical studies involve full circumferential repair, where clinicians generally perform inlay repair [127]. The most likely reason is that preclinical investigators endeavoring to demonstrate the efficiency of the experimental therapy for the most challenging in circumferential techniques, supposing that it could also be effective in less difficult in inlay methods [126].

Fibrotic bladder model

Most in vivo experimental models of tissue-engineered bladders are performed in the normal bladder following cystectomy. However, in clinical situations, patients have diseased bladders with fibrosis, chronic inflammation, or lack of good blood supply. Thus a disease model that mimics the target disease conditions is needed to test the efficacy of tissue engineering technology. Several approaches have been used to create a neurogenic bladder dysfunction model [128] or fibrotic bladder model induced by bladder atrophy due to urinary diversion [129], bladder ischemia [130], or chemical agents [131].

To generate a bladder atrophy model, urinary diversion is performed to achieve a rapid decrease in contractile function in adult or fetal animal bladders. In one study, Liu

TABLE 46.5 Criteria for tissue engineering techniques for lower urinary tract regeneration.

Preimplantation	
Cells	• Characterize cell proliferation capacity, phenotypes, paracrine effects of stem cells, urothelially and myogenically differentiated stem cells or cultured urothelial and smooth muscle cells with immunocytochemical staining; label to monitor the fate of grafted cells if needed
Scaffold	• Determine biocompatible, biodegradable and mechanical properties, porosity, pore size, and bioactive proteins within scaffold (for natural collagen-based extracellular matrix biomaterials)
Cell-biomaterial interaction	• Demonstrate interactions for cell adhesion and infiltration into the scaffold using histologic techniques
Postimplantation	
Gross examination	• Graft size and hardness, tissue adhesion around organs • Signs of graft contraction, stoned, or calcification on the bladder lumen side
X-ray examination	The anatomy of low urinary tract can be visualized with the use of X-ray contrast media. A cystogram is used for the bladder and a urethrogram for the urethra. A urethrogram can be done in conjunction with a cystogram or as a separate procedure
Bladder function tests	• Bladder volume, pressure, and contraction style with urodynamic • Contractility of regenerated bladder strips assessed with organ bath
Histologic structure	• Cell survival rate • Distribution and life span of the labeled graft cells • Entirety of urothelial layers and thickness of muscle layers • Ratio of collagen and muscle in regenerated bladder tissue • Angiogenesis or revascularization and innervation

et al. [129] developed a urinary diversion model in the rat. Female Sprague Dawley rats were distributed into age-matched control, sham urinary diversion, and urinary diversion groups. Each group was subsequently evaluated 1 or 8 weeks after urinary diversion or sham operation. The diversion was made by the surgical disconnection of the ureters from the bladder and implantation to the uterine cervix. The results demonstrated that bladder weight decreased in the diversion group. The urinary diversion group had decreased intercontractile intervals and voided volumes compared to the control and sham-operated groups. Compliance was decreased in bladders of rats with urinary diversion. Smooth muscle and urothelium were decreased as a proportion of the total bladder cross-sectional area. Collagen increased in rats with either 1 or 8 weeks of urinary diversion versus controls. Urinary diversion caused decreased expression of muscarinic 3 and ligand-gated purinergic 1 receptor, but no change in muscarinic 2 or ligand-gated purinergic 2 receptors. In summary, urinary diversion causes dysfunctional and abnormal morphometric alterations in the bladder in this model.

In a study by Matsumoto et al. [132], urinary diversion was performed in fetal sheep after 90 days of gestation (term 147 days), and bladder tissue was obtained 2 weeks later. Bladders from fetal sheep subjected to urinary diversion weighed significantly less than control fetal bladders. Marked reorganization of smooth muscle elements was observed in those with diversion. Carbachol stimulated a tonic contraction, while field stimulation administered during the tonic contraction elicited a phasic relaxation or a biphasic response, consisting of an initial relaxation and then a phasic contraction in control and diverted bladders. Contractile responses of defunctionalized strips to carbachol were significantly less than those of control bladder strips. In brief, urinary diversion in normal fetal sheep resulted in marked bladder atrophy, reduced muscle mass, and decreased bladder contractility.

Both indwelling urethral catheterization and suprapubic catheterization are types of urinary diversion. The former is associated with a risk of urolithiasis, urethral trauma, urethral erosions and/or strictures, bladder fibrosis, epididymitis, orchitis, and bladder cancer. Therefore in most clinical cases, indwelling urethral catheters are inappropriate for long-term treatment, and suprapubic catheters are a better option. But recent clinical data [133] showed that long-term indwelling urinary catheterization has similar rates of upper tract damage, vesicoureteral reflux, renal or bladder calculi, and symptomatic urinary tract infections compared to suprapubic catheters. The catheter was changed every 2 weeks, which can prevent the complications of indwelling urethral catheterization. Although a bladder dystrophy model is closer to diseased conditions, it is time-consuming to create such a model. A novel bladder fibrosis model is needed for bladder tissue regeneration studies.

Clinical trials

Clinical translation

Human UC and muscle cell can be expanded in vitro, seeded onto polymer scaffolds, and allowed to attach and form sheets of cells for the lower urinary tract reconstruction. The cell-polymer scaffold can then be implanted in vivo. Histologic analysis indicated that viable cells were able to self-assemble back into their respective tissue types and would retain their native phenotype [134]. These experiments demonstrated, for the first time, that composite layered tissue-engineered structures could be created de novo. Before this study, only nonlayered structures had been created in the field of regenerative medicine.

In order to determine the effects of implanting engineered tissues in continuity with the lower urinary tract, animal models of bladder augmentation were used [135]. Partial cystectomies were performed in dogs. The animals were divided into two experimental groups. One group had their bladder augmented with a nonseeded bladder-derived collagen matrix, and the second group had their bladder augmented with a cell-seeded construct. The bladders augmented with matrices seeded with cells showed a 100% increase in capacity compared with bladders augmented with cell-free matrices, which showed only a 30% increase in capacity.

Most of the free grafts (without cells) used for bladder replacement in the past showed adequate histology, in terms of a well-developed urothelial layer, but they were associated with an abnormal muscular layer with varying tissue development. It has been well established for decades that the bladder is able to regenerate generously as urothelium is associated with a high reparative capacity. Bladder muscle tissue is less likely to regenerate in a normal fashion. Both urothelial and muscle ingrowth are believed to be initiated from the edges of the normal bladder moving toward the region of the free graft [136]. However, contracture or resorption of the graft has been evident in most cases. The inflammatory response toward the matrix may contribute to the resorption of the free graft. It was hypothesized that building 3D structure constructs in vitro, before implantation, would facilitate the eventual terminal differentiation of the cells after implantation in vivo and would minimize the inflammatory response toward the matrix, thus avoiding graft contracture and shrinkage. A dog study demonstrated a major difference between matrices used with autologous cells (tissue-engineered matrices) and those used without cells [135]. Matrices implanted with cells for bladder augmentation retained most of their implanted diameter, as opposed to matrices implanted without cells for bladder augmentation, in which graft contraction and shrinkage occurred. The histomorphology demonstrated a marked paucity of muscle cells and a more aggressive inflammatory reaction in the matrices implanted without cells. Epithelial−mesenchymal signaling is important for the differentiation of bladder smooth muscle [137]. The results of initial studies showed that the creation of artificial bladders might be achieved in vivo; however, it could not be determined whether the functional parameters noted were caused by the augmented segment or by the intact native bladder tissue. To better address the functional parameters of tissue-engineered bladders, an animal model was developed that required a subtotal cystectomy with subsequent replacement with a tissue-engineered organ [138].

The subtotal cystectomy reservoirs that were not reconstructed and the polymer-only reconstructed bladders showed a marked decrease in bladder capacity (22%-46% of pre-operative values) and compliance (10% and 42% total compliance). Average bladder capacity was increased in the cell seeded tissue engineered bladder replacements (95% of the original pre-cystectomy volume). The compliance of the cell-seeded tissue-engineered bladders showed almost no difference from preoperative values that were measured when the native bladder was present (106%). Histologically, the nonseeded scaffold bladders presented a pattern of normal UCs with a thickened submucosal fibrotic and a thin layer of muscle fibers. The retrieved tissue-engineered bladders showed a normal cellular organization, consisting of a trilayer of urothelium, submucosa, and muscle. Immunocytochemical analyses confirmed the muscle and urothelial phenotype. S-100 staining indicated the presence of neural structures [138]. These studies, performed with PGA-based scaffolds, have been repeated by other investigators, showing similar results in large numbers of animals long term [139]. The strategy of using biodegradable scaffolds with cells can be pursued without concerns for local or systemic toxicity [140]. However, not all scaffolds perform well if a large portion of the bladder needs replacement. In a study using SIS for subtotal bladder replacement in dogs, both the unseeded and cell-seeded experimental groups showed graft shrinkage and poor results [141]. The type of scaffold used is critical for the success of these technologies. The use of bioreactors, wherein mechanical stimulation is started at the time of organ production, has also been proposed as an important parameter for success [142,143].

To evaluate the effect of cell-seeded tissue engineering technology in the bladder regeneration compared to scaffold alone, a group of experimental dogs underwent a trigone-sparing cystectomy and were randomly assigned to one of three groups. One group underwent closure of the trigone without a reconstructive procedure, another group underwent reconstruction with a nonseeded bladder-shaped biodegradable scaffold, and the last group underwent reconstruction using a bladder-shaped biodegradable scaffold that was seeded with autologous UC and SMC

[138]. The cystectomy-only and nonseeded controls maintained average capacities of 22% and 46% of preoperative values, respectively. Average bladder capacity of 95% of the original precystectomy volume was achieved in the cell-seeded tissue-engineered bladder replacements; however, the subtotal cystectomy reservoirs that were not reconstructed and the polymer-only reconstructed bladders showed a marked decrease in bladder compliance (10% and 42% of total compliance, respectively). The compliance of the cell-seeded tissue-engineered bladders was almost no different from preoperative values (106%). Histologically, the nonseeded scaffold bladders presented a pattern of normal UCs with a thickened submucosal fibrotic and a thin layer of muscle fibers. The retrieved tissue-engineered bladders showed normal cellular organization, consisting of a trilayer of urothelium, submucosa, and muscle [138], indicating the benefit of cell-seeded tissue engineering technology in the bladder reconstruction, compared to nonseeded tissue-engineered bladder.

For urethral reconstruction, many surgical procedures, such as autografting to replace damaged areas of the male urethra, may eventually fail. Various strategies have been proposed over the years for the regeneration of urethral tissue in several animal models, including woven meshes of synthetic polymers such as PGA without cells [144,145] and with cells [7], naturally derived collagen-based materials such as decellularized bladder submucosa [106], acellular urethral submucosa [146], and small intestine submucosa [124].

Clinical studies

A clinical experience involving engineered bladder tissue for cystoplasty reconstruction was conducted starting in 1998. A small pilot study of seven patients was reported [5], using a collagen scaffold seeded with cells with or without omental coverage or a combined PGA−collagen scaffold seeded with cells and omental coverage. The patients reconstructed with the engineered bladder tissue created with the PGA−collagen cell-seeded scaffolds showed increased compliance, decreased end-filling pressures, increased capacities, and longer dry periods over time [5]. It is clear from this experience that the engineered bladders continued their improvement with time, mirroring their continued development. Although this report was promising in terms of showing that engineered tissues can be implanted safely, it is just a start in terms of accomplishing the goal of engineering fully functional

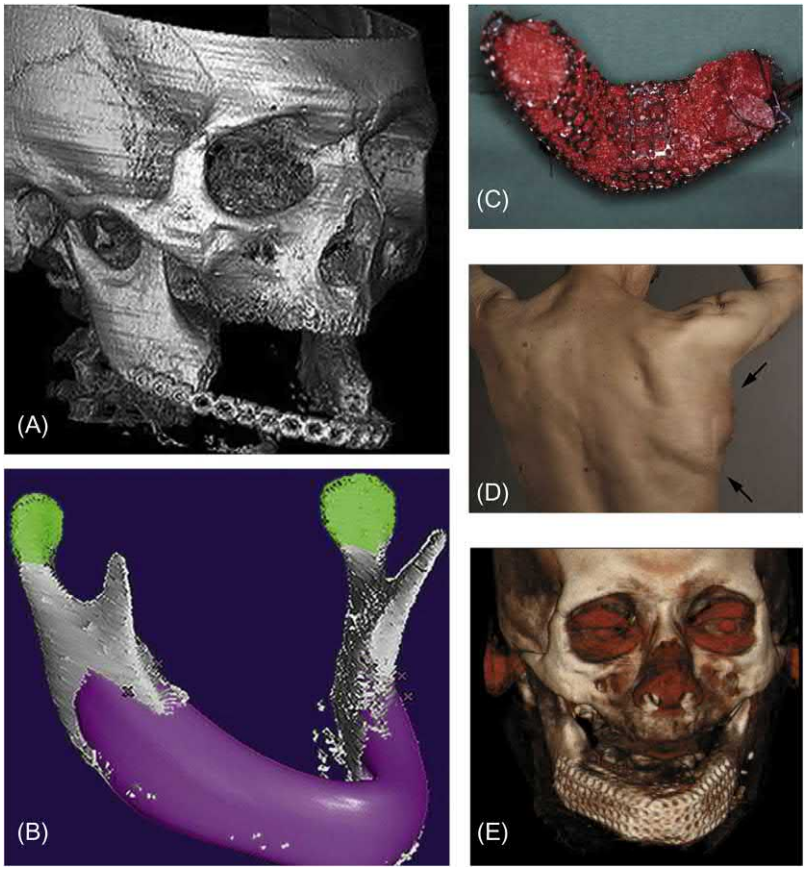

FIGURE 46.1 Image-guided tissue-engineered reconstruction of a massive mandibular defect. (A) The region of interest (jaw) is imaged using 3D CT. (B) The CT data are then fed to CAD software to generate an idealized virtual replacement of the missing parts of the mandible. (C) A titanium mesh is then formed in the shape of the missing bone model and augmented with BioOss hydroxyapatite blocks, OP-1 collagen implant, rhBMP-7, and autologous bone marrow aspirate. (D) The engineered mandibular graft is implanted in a heterotopic muscular pouch in the patient to establish vascularization and initial osteogenesis. (E) The graft was finally implanted orthotopically to reconstruct the mandibular defect. The patient had functional mastication and satisfactory esthetic outcome. *3D*, Three-dimensional; *CAD*, computer-aided design; *CT*, computed tomography. *Reproduced with permission from Warnke et al. (2004).*

bladders. This was a limited clinical experience, and the technology is not yet ready for wide dissemination, because further experimental and clinical studies are required. FDA Phase II studies have now been completed.

The effectiveness of using collagen-based inert matrix was confirmed clinically in a series of four patients with a history of failed hypospadias reconstruction. Twenty-two-month follow-up showed successful repair of urethral defects with human bladder acellular collagen matrix in three out of four patients [147]. In another study of 30 patients with recurrent stricture disease, human demineralized bone matrix obtained from cadaveric donors was processed and prepared for use as an off-the-shelf material [148]. Up to 36-month follow-up showed that a healthy urethra bed (two or fewer prior urethral surgeries) was needed for successful urethral reconstruction using the acellular collagen-based grafts. In an advanced study, with more than 10-year follow-up [149], a human clinical trial with a collagen-coated PGA tubularized scaffold seeded with autologous cells was conducted in a pilot series of five patients. The scaffolds were seeded with autologous cells derived from bladder biopsies taken from each patient. The cell-seeded scaffolds were then matured in an incubator and used to repair the urethral defects present due to trauma. Long-term follow-up evaluation showed normal range urinary flow rates, and voiding cystourethrograms indicated that these patients maintained wide urethral calibers. Yearly biopsies showed that the grafts had a histological architecture consistent with normal urethral tissue (Fig. 46.1).

Conclusion

We reviewed experimental and clinical data related to bladder and urethra regeneration, highlighting the use of different stem cell types and various biomaterials. Recent progress suggests that engineered urologic tissues may have wide use for clinical applicability. Assessments of the histologic structure and physiologic function of the lower urinary tract can help better elucidate mechanisms responsible for functional tissue-engineered bladders and urethra. Well-established in vitro and in vivo models are available for experimental evaluations of the regenerated bladder and urethra, providing invaluable data to predict clinical efficacy.

Standard cell culture studies can define the biological and molecular cues of UC, SMC, and endothelial cell or differentiated stem cells. Novel and noninvasive cell sources are needed to further improve the regenerative efficacy of the lower urinary tract. In addition, 3D construction remains critical to recapitulate the epithelial−stromal microenvironment in bladder and urethra regeneration studies. The development and optimization of reliable and reproducible scaffolds with the necessary porosity, biodegradability, flexibility, and firmness are vital for assessing in vitro and in vivo efficacy of tissue-engineered bladders and urethra. Biomaterials with coated with growth factors are promising tools for bladder and urethra regeneration. Therapeutic investigations should be continued, with the development of new biomaterials and optimized cell source to improve treatment outcomes for the lower urinary tract diseases through the tissue engineering technology.

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Chapter 47

Tissue engineering for female reproductive organs

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Introduction

Fertility is a key element of reproductive health. The World Health Organization recognizes infertility as a global public health issue, affecting between 8% and 12% of reproductive-aged couples [1]. By definition a couple is considered infertile when they fail to conceive after 1 year or longer of timely unprotected intercourse [2,3]. For most women the experience of infertility negatively affects multiple aspects of their quality of life, causing substantial sociopsychological burdens [4].

Female infertility may arise from genetic abnormalities, infectious or environmental agents, endocrine imbalances, aging, and specific acquired diseases [5]. The prevalence of infertility, secondary to genetic abnormalities, ranges from 0.1% to 5% of the general female population [6–9]. These developmental issues can be associated with Müllerian duct malformations such as the Mayer–Rokitansky–Küster–Hauser syndrome (MRKHS). It is characterized by an absent uterus, cervix, and upperpart of the vagina and occurs in approximately 1 of 4000 newborn girls [10–12]. Acquired diseases associated with female infertility may result from fallopian tubes dysfunction/obstruction, endometriosis, leiomyomas (fibroids) distorting the uterine cavity, severe intrauterine adhesions, and hysterectomy [13,14]. The most prevalent etiologies underlying female infertility include ovulatory disorders, fallopian tube occlusion, and endometriosis.

Reproductive medicine has experienced significant advances over the past decades with the development of assisted reproductive technologies, such as in vitro fertilization, and molecular and genetic approaches to female infertility [15]. However, there is an increased demand for therapies that will restore function and/or compensate for the anatomical absence of organs. Tissue engineering approaches have emerged as an attractive alternative to address these problems by integrating biomaterials, cells, and growth factors to promote functional tissue repair. This chapter highlights progresses in tissue engineering applications for female reproductive organs.

Uterus

The uterus is the largest organ of the female reproductive system. It has an inverted pear shape and is anatomically divided into the uterine body or fundus connected on each side to the fallopian tubes; and the lower segment where the cervix opens into the vaginal cavity. Its histological elements include the endometrium (inner epithelial layer surrounded by stroma); the myometrium, consisting of oriented layers of smooth muscle cells; and outer serosa (perimetrium) that envelop the organ.

Uterine factor infertility affects 2%–8% of reproductive-aged women and arises from congenital (MRKHS, uterine hypoplasia, uterine malformation) or acquired causes including hysterectomy due to benign diseases (leiomyoma, adenomyosis), postpartum hemorrhage, intrauterine adhesions (Asherman's Syndrome), or malignant tumor [16,17]. Currently, these patients can achieve motherhood through gestational surrogacy and/or adoption; however, both options are associated with complex legal and ethical issues [18–20].

Allogenic uterus transplantation has been investigated preclinically and clinically as a potential curative therapy for uterine infertility [21,22]. The first live birth from a transplanted uterus was reported in 2014 by Brännström et al. [23]. While considered a promising approach, it is still experimental and associated with significant limitations, including organ donor shortage and a need for long-term immunosuppression therapy [24].

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Acellular tissue engineering approaches for uterine tissue repair

Current tissue engineering approaches involve the use of constructs for partial uterine tissue repair. One of the first attempts in uterine tissue regeneration investigated the use of synthetic graft materials, including polytetrafluoroethylene (PTFE) and polyetherurethane/poly-L-lactide (PU/PLLA), in a rat model [25]. Initial experiments using these synthetic polymers showed that 6 weeks after engraftment, nonabsorbable PTFE grafts elicited an intense local inflammatory response, leading to poor tissue ingrowth and severe adhesions. While endometrium-like tissue ingrowth was observed within biodegradable PU/PLLA grafts, they failed to provide luminal patency.

Naturally derived biomaterials, including collagen and extracellular matrices (ECMs), have been investigated as candidate scaffold material for uterine tissue engineering due to their biodegradability and biocompatibility properties. Collagen scaffolds, loaded with basic fibroblast growth factor (bFGF), were developed by fusing a collagen-binding domain (CBD) to the N-terminal of native bFGF (NAT) and tested in a rat model of severe uterine injury [26]. A section of the uterine horn was removed and replaced by a 15 mm × 5 mm collagen-based bFGF delivery system membrane. At 90 days after surgical implantation, there was enhanced vascularization, improved endometrium ingrowth, and partial development of smooth muscle bundles where there had once been collagen-based implants. In addition, postoperative pregnancy rate in the collagen/CBD−bFGF implant group was comparable to the sham-operated group [26].

Using a similar approach, vascular endothelial growth factor (CBD−VEGF)-bound collagen was injected in a full-thickness rat uterine injury model [27]. The results showed that the collagen targeting VEGF delivery system promoted tissue ingrowth, neovascularization, and endometrial gland development in the injection sites [27].

ECMs are thought to be advantageous over synthetic scaffolds due to their distinct native tissue microstructure and composition (collagen and glycosaminoglycans). They also include the presence of several growth factors, which could potentially render an adequate cell differentiation and growth, as well as constructive host tissue remodeling response in vivo [28]. The specific three-dimensional (3D) features, composition, and distribution of the ECM elements may vary depending on the tissue source and processing methods [29]. Initial studies applying ECM-derived scaffolds for uterine tissue regeneration involved the use of porcine small intestinal submucosa (SIS) in rabbits [30]. The results indicated tissue ingrowth within engrafted membranes, but functionality was limited due to poor mechanical strength.

ECM-based scaffolds for uterine tissue reconstruction can be obtained through the process of decellularization (DC) of tissues and organs. The commonly used DC processes involve physical, chemical, and enzymatic methods [31]. As an initial step, Santoso et al. [32] investigated the effectiveness of three different DC protocols using Sprague Dawley rat uterine segments. Protocols involved the use of sodium dodecyl sulfate (SDS), high hydrostatic pressure (HHP), or Triton-X at optimized conditions and examined their effects on in vivo uterine tissue regeneration. Histological results indicated efficiency upon cell removal and minimal changes in the overall matrix structure using SDS and HHP protocols, whereas Triton-X severely reduced collagen and elastin content within the matrix. Thirty days after decellularized grafts (15 × 5 mm) were implanted into rat uteri, there was greater cell migration and tissue integration in the HHP group versus the other groups; however, it was restricted to the native tissue-graft transition area due to the absence of a connected microvascular network to support nutrition [32]. To address the lack of vascular supply and nutrient delivery to cells, whole uterus perfusion-based DC techniques have been developed in murine [33] and porcine models [34]. In vitro and in vivo studies have been conducted to optimize the applicability of whole uterus ECM-based scaffolds for partial uterine tissue repair.

Cell-seeded scaffolds for partial uterine repair

In an effort to enhance the regenerative capacity of tissue-engineered scaffolds, different cell sources combined with biomaterials have been explored. Atala et al. investigated the possibility of engineering autologous uterine tissue using a biodegradable polymer-based scaffold seeded with primary uterine cells [35,36]. Endometrium and myometrium cells were isolated and expanded ex vivo from rabbit uterine horns and seeded in a stepwise fashion onto the lumen and outer surface, respectively, of preconfigured semicircular scaffolds composed of polyglycolic acid (PGA)—coated poly-DL-lactide-*co*-glycolide (PLGA). The uterine construct was used to replace a subtotal excised horn in animals from which the cells derived. Six months after cell-seeded construct implantation, the neouterine tissue showed organized cellular and anatomical structures as well as expression of specific markers for epithelial, stroma, and smooth muscle cells. Functional studies are being conducted to assess the reproductive outcomes from the autologous bioengineered uteri.

Collagen scaffolds seeded with donor rat bone marrow−derived mesenchymal stem cells (BM-MSCs) have been studied in a murine model of severe uterine

injury [37]. A suspension of labeled BM-MSCs (5×10^5 cells/cm^2 scaffold) was seeded into collagen membranes (15×5 mm) and incubated for 1−3 hours before being engrafted in a partially removed uterine horn. Four weeks after surgery, BM-MSCs migrated to the basal layer of the graft, and the perigraft tissue had greater expression of bFGF, insulin-like growth factor 1, VEGF, and transforming growth factor-β1 than that in the collagen membrane-only group. Reproductive studies conducted 90 days after engraftment confirmed viable offspring in the BM-MSC/scaffold group suggesting that BM-MSCs may contribute to functional uterine tissue regeneration [37].

In another study, collagen scaffolds were loaded with endometrium-like cells derived from human embryonic stem cells (ESCs) and used as an implant in a rat uterine full-thickness injury model [38]. The results from in vivo studies indicated that although the cellularized collagen scaffold enhances uterine tissue regeneration, few human ESCs-derived cells were detected in the engrafted sites.

Other DC/recellularization techniques have been applied to create implantable grafts for uterine tissue regeneration. Miyazaki et al. [39] attempted to repopulate a whole rat decellularized uterine matrix injecting 5.1×10^7 neonatal uterine cells, 2.7×10^7 adult uterine cells, and 1.0×10^6 rat BM-MSCs in the uterine wall. After 3 days of incubation in a perfusion system, an endometrium-like tissue formation was observed, although cells were not evenly distributed within the matrix. When recellularized grafts (15×5 mm) were implanted in a partially excised rat uterine horn, uterine tissue ingrowth was noticed within the grafts, and pregnancy outcomes were reported to be nearly comparable to animals with an intact uterus. In another study, Hellström et al. [40] recellularized whole rat uterus matrices with donor primary heterogeneous uterine cells mixed with rat green fluorescent protein (GFP)-labeled MSCs. Grafts (20×5 mm) received multiple injections of a cell mix (one primary uterine cell per 150 GFP-MSCs) and were incubated for 72 hours. In vitro analysis of recellularized scaffolds revealed cell distribution limited to the matrices' external surface. Recellularized matrices (5×10 mm) were implanted in full-thickness excised rat uterine horns; and tissue ingrowth within the grafts was described at 6 weeks, mainly from host cells. Pregnancies in the remodeled uterine horn were reported, although embryo implantation did not occur directly in the grafts [40].

Scaffold-free approaches for partial uterine repair

Scaffold-free approaches have been evaluated in uterine tissue engineering to produce tissues by mimicking developmental processes. These methods often follow a pattern of cell condensation, cell proliferation, cell differentiation, ECM production, and tissue maturation. Campbell et al. [41] implanted tubular boiled blood clot templates (25×5 mm) in the peritoneal cavity of rats to produce autologous grafts. After 2 weeks an encapsulated myofibroblasts-rich tissue was formed and used to partially replace one uterine horn of the host animal. At 12 weeks the grafted tissue developed organized morphology with luminal and glandular epithelium, and two distinct layers of smooth muscle bundles interspersed with collagen, resembling native tissue; moreover, engrafted horns supported late-stage pregnancies.

Cell sheet engineering technology has also been explored for endometrium reconstruction. This technique involves cells self-organization and requires external manipulation to form a desired structure. Primary endometrial cells were isolated from immature mice, and rats were cultured using a temperature-responsive culture system [42]. Murine endometrial cell sheets harvested by lowering the culture temperature were transplanted onto exteriorized muscle and formed endometrium-like tissue structures.

Uterine cervix tissue engineering

The cervix has important mechanical and protective roles during pregnancy and undergoes significant remodeling during parturition. A dysfunctional cervix may result in preterm birth, which is associated with significant perinatal complications. House et al. [43] developed 3D cervical−like tissue constructs to investigate cervical remodeling in vitro. Human stromal cervical cells were seeded on a collagen-coated porous silk protein scaffold and cultured under static or dynamic conditions. At 8 weeks, cervical cells proliferated and synthetized an ECM with biochemical and structural properties that resembled native tissue. The dynamic culture condition led to increased collagen deposition, glycosaminoglycan synthesis, and mechanical stiffness of the 3D cervical construct.

Ovary

The hypothalamic−pituitary−ovarian axis controls mechanisms involved in the development and regulation of female reproductive function. The fundamental role of the ovary is to produce oocytes capable of fertilization as well as secrets sex steroids hormones. The ovarian follicle is the functional unit of the ovary, and it comprises an oocyte (germ cell) surrounded by layers of somatic follicular cells (granulosa and theca cells) and a basement membrane. Ovarian hormones play a major role in breast development, uterine receptivity for embryo implantation, bone homeostasis, as well as sexual function [44].

Pathological ovarian dysfunction may occur due to surgical resection, premature ovarian failure, or cancer chemotherapy treatment, compromising reproductive function and leading to an increased risk for cardiovascular diseases and osteoporosis. Current ovary tissue engineering approaches aim to restore its gonadal and endocrine physiological roles.

Tissue engineering ovarian follicles

In vitro culture systems to promote ovarian follicle growth and maturation have been proposed as a promising strategy to restore female fertility. Maintenance of the complex 3D architecture and granulosa–oocyte interaction is critical for successful in vitro follicle maturation. In conventional two-dimensional (2D) tissue culture systems, early-stage follicles tend to flatten, and granulosa cells migrate away from the oocyte, impairing the maturation process [45–48]. To address 2D-related limitations, tissue engineering technologies have been applied to develop in vitro maturation systems to recapitulate the architecture of ovarian follicles and provide a supportive environment for cell–cell interaction and paracrine signaling to direct follicular growth [49,50]. To enhance ovarian graft survival, Shikanov et al. [51] developed a delivery system combining hydrogels (fibrin and alginate) and angiogenic factors in a murine model. A cryopreserved/thawed ovarian cortical tissue was encapsulated in fibrin hydrogel modified with heparin-binding peptide loaded with VEGF and transplanted into the ovarian bursal cavity. The results indicated that the delivery strategy enhanced neovascularization and improved survival of primordial follicles in the graft. In another study, Krotz et al. [52] developed a self-assembled artificial human ovary as a tool to mature early antral oocytes in vitro. In this study, theca cells were isolated from reproductive-aged women and cultured into agarose molds to form a honeycomb structure. Granulosa cell spheroids or cumulus–oophorus complexes (COCs) were then placed in the honeycomb openings. After 72 hours, theca cells surrounded the COCs, resulting in self-assembled complex microtissues that supported oocyte maturation, confirmed by visualization of the polar body extrusion.

Hydrogel-based systems for in vitro maturation of isolated oocytes have also been explored. Pangas et al. [53] pioneered the use of an alginate-based system to grow individual immature mouse granulosa cell–oocyte complexes. Alginate is a naturally occurring polysaccharide, typically extracted from brown algae, and one of the most commonly used biomaterials for encapsulation owing to its biocompatibility, low toxicity, low cost, biomechanical properties, and its ability to form gel in the presence of calcium ions [54,55]. Studies using 3D alginate culture systems reported oocytes in vitro maturation [56,57]; moreover, embryos derived from in vitro fertilized cultured oocytes [58] produced viable offspring [59].

Cell encapsulation techniques have also been investigated as a potential cell-based hormone replacement therapy. The advantage of this approach over current pharmacological therapies is to potentially provide a more physiological activation of the hypothalamic–pituitary–ovarian axis in patients with ovarian failure. Ideally, the microcapsules should be biocompatible, provide a barrier between the allogeneic ovarian cells and the recipient to prevent immune rejection, and promote long-term cell survival. Sittadjody et al. [60] developed an engineered multilayer ovarian tissue model using alginate-based encapsulated ovarian cells from immature rats that secretes ovarian hormones in response to follicle-stimulating hormone and luteinizing hormone in vitro. Moreover, these 3D-engineered multilayered ovarian constructs achieved stable hormone secretion for up to 90 days following in vivo implantation in ovariectomized rats [61]. Similarly, studies using ovarian cell microcapsule delivery systems have reported increased levels of endogenous female hormones following transplantation in rodent models [62,63]. Ovarian cell encapsulation techniques have also been applied in microfluidic devices to create a biomimetic ovarian microtissue [64]. These systems have the potential to recapitulate human ovarian folliculogenesis [65] and therefore further improve in vitro maturation systems for fertility restoration in young cancer survival patients.

Vagina

Vaginal agenesis and other congenital or acquired disorders compromise a woman's sexual and reproductive life, often resulting in psychological issues [66,67]. A variety of vaginoplasty techniques have been described to create a neovaginal canal between the bladder and rectum. However, current approaches using nonvaginal autologous and allogenic grafts to line the surgically created cavity have been associated with complications such as graft rejection, chronic stenosis, and poor mechanical strength [68].

Tissue engineering approaches for neovagina reconstruction

The lack of optimal biomaterials that adequately mimic the vaginal tissue structure has led researchers to develop alternative vaginal tissue substitutes for neovaginal reconstruction procedures. Proof-of-concept studies evaluated the applicability of synthetic scaffolds for vaginal reconstruction in animal models [69–71]. Biodegradable PGA/PLGA scaffolds seeded with in vitro expanded autologous vaginal epithelial and smooth muscle cells were

subcutaneously implanted in nude mice [70]. After 4 weeks, retrieved grafts were neovascularized and had multilayered vaginal tissue−like organization.

Biologic scaffolds such as acellular vagina matrix (AVM) have been proposed as a potential source of organ-specific biomaterial [72]. Porcine vagina tissues were decellularized using a multistep method, and the resulting matrices displayed biomechanical properties and growth factor content comparable to that of the SIS. In vivo AVM biocompatibility was tested in a rat model of vaginal reconstruction. Three months after implantation, the neovaginal tissue formed epithelial and muscle layers.

Stem cell sources have also been investigated for autologous vaginal tissue engineering. BM-MSCs cocultured with vaginal epithelial cells were seeded onto SIS matrix for rat vagina reconstruction [73]. In vivo results indicated epithelial differentiation of seeded cells and significant matrix infiltration in the graft.

The potential of autologous adult cells sources has also been evaluated in clinical studies. Autologous vaginal vestibule cells were isolated and used to fabricate grafts in patients with MRKHS [74,75]. Cells were in vitro expanded and mounted on hyaluronic acid-embedded gauze to cover the surface of a surgically created vaginal canal. After a 12-month follow-up, neovaginal tissue biopsies revealed vaginal-like tissue formation, and patients reported a satisfactory sexual quality of life [75].

A variety of scaffold materials for vaginal reconstruction have been investigated in clinical studies, including acellular dermal matrix [76], SIS [77−80], allogenic amniotic membrane [81,82], and oxidase cellulose [83,84]. Raya-Rivera et al. [85] reported that tubular-shaped SIS scaffolds seeded with autologous primary vulvar cells are a feasible method for neovaginal reconstruction in patients with MRKHS. SIS membranes initially measuring 7×10 cm were customized for each patient based on their pelvic morphometric analyses and seeded with ex vivo cultured epithelial and smooth muscle cells. SIS-engineered grafts were then surgically implanted and serial tissue biopsies, imaging, and clinical exams confirmed a stable lining with native-like three-layered structures and patent neovaginal cavity for up to 8 years. Moreover, patients were sexually active, and a validated self-administered Female Sexual Function Index questionnaire indicated had functional variables were within the normal range.

Conclusion and future perspectives

Tissue engineering approaches have demonstrated great potential for treating disorders in the female reproductive system. Experimental studies provide encouraging results for uterine tissue engineering strategies for partial uterine repair. Several cell sources and promising scaffold materials and designs have been established, but there is no consensus or standardized methods to effectively produce engineered uterine constructs. In vivo studies suggest that cell migration and positioning within ECM-based scaffolds are primarily dependent on microstructural and microenvironment factors [86]. Additional studies are needed to produce adequately vascularized grafts that mimic the complex microarchitecture and plasticity of the human uterus and reproduce the complex nature of uterine function.

Progress in 3D culture systems for the follicle-enclosed oocyte maturation represents a major achievement in reproductive biology. Challenges still remain regarding the optimization of these in vitro culture systems for growing follicles with greater diametric sizes such those present in human ovaries.

The first-in-human study of autologous-engineered vaginas provides compelling evidence of safety and feasibility of this technology. Nonetheless, clinical trials comparing the long-term postoperative performance of various scaffold materials for vaginal tissue reconstruction are paramount to providing strong evidence of successful outcomes before introducing new technologies into the clinical setting.

Lastly, the future of tissue engineering approaches for the human female reproductive organs will rely on identifying optimal scaffold materials, viable cell sources, and comprehension of the underlying mechanisms of organogenesis to achieve engineer reproductive tissues that are functional and safe for recipients and their offspring.

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Chapter 48

Male reproductive organs

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Introduction

The science of male reproductive function and dysfunction has experienced significant progress in recent years. Especially clinical applications of molecular medicine to male infertility and hypogonadism [1], the "invention" of intracytoplasmic sperm injection (ICSI) as a treatment of male infertility [2] and the introduction of effective oral medication for erectile dysfunction (ED) [3,4] can be considered major breakthroughs. Infertility affects around 15% of couples who wish to have children, and half of these cases are associated with male-related factors [5]. ED is defined as the consistent or transient disorder, and inadequate erection affects at least half of men between the ages of 40 and 70, while chronic ED affects about 5% of men in their 40s and up to 25% of men by the age of 65 [6,7]. Hormonal and surgical approaches have been used to treat patients suffering from reproductive system disorders. Currently, regenerative medicine has become a promising concept in the development of new therapies for all fields of medicine, including reproduction [8]. The field of tissue engineering and regenerative medicine covers different areas of biomedical technology, including biomaterials, tissue engineering, and stem cell therapy. Three main strategies have been used: (1) utilizing extracellular matrices (ECMs) without cells, allowing the natural ability of the body to generate new tissue; (2) utilizing ECM seeded with cells for implantation; and (3) direct injection of cells either with or without carriers such as hydrogels. The ECMs used for tissue engineering may either be synthetic or derived from natural sources [9,10]. Different cell types may also be used, including (1) tissue-specific stem cells; (2) mesenchymal stem cells (MSCs); and (3) pluripotent stem cells such as embryonic stem cells, induce pluripotent stem cells, or amniotic and placental-derived stem cells. These cells can be either autologous or allogeneic. Autologous cells derived from a small biopsy are the preferred source because of immune compatibility. The biopsy is obtained from the patient, and the cells isolated from this biopsy tissue are expanded in vitro. However, for some patients, this is not an option as there is not enough functional tissue to obtain a biopsy. Therefore other sources of cells, such as stem cells (either autologous or allogeneic), might be used for these patients [9,10]. The ultimate goal of regenerative medicine strategies for the treatment of reproductive system disorders is to restore normal sexual function and preserve fertility.

Testes

The hypothalamus, the pituitary, and the testes form an integrated system responsible for an adequate secretion of male hormones and for normal spermatogenesis. The general anatomy of the testis consists of the seminiferous tubules and, among them, the interstitial space [11]. The seminiferous tubules contain germ cells in different maturation levels. The pool of germ cells is supported by spermatogonial stem cells (SSCs) that are covered by a layer of Sertoli cells embedded in lamina propria [12]. The lamina propria consists of the basal membrane covered by peritubular cells. Differentiated germ cells including spermatocytes and spermatids are located across the tubule toward its center. The main component of the interstitial space is the Leydig cell population that produces testosterone, but the interstitial space also contains macrophages, lymphocytes, loose connective tissue, and neurovascular bundles [13].

Spermatogonial stem cell technology

Etiologies of nonobstructive azoospermia include endocrinopathy, testicular failure, genetic anomaly, and idiopathic factors. In the United States on average, about five

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men become azoospermic everyday due to the adverse effect of cancer treatments [14]. In sexually mature boys and men suffering from cancer and who are at risk of infertility, sperm cryopreservation is the simplest and most effective method to preserve their fertility [15]. However, less than 25% of these patients apply for sperm banking [16]. Lack of knowledge about semen cryopreservation is one of the major reasons of low percentage of sperm storage [16]. However, since the production of sperm has not yet started in prepubescent boys [17], sperm banking is not an option for prepubertal cancer patients. On average, more than 14 boys younger than 15 years of age are diagnosed with cancer every day and more than 80% of them will be cured [18]. Therefore long-term infertility will be a critical issue for many of these childhood cancer survivors that have no option for sperm banking. SSCs are present in the testis of most mammalian species, including nonhuman primates and human [19]. Spermatogonia are the cells located in the basement membrane of seminiferous tubules in the testis, and a small subpopulation of them are SSCs that play a primary role in spermatogenesis [19]. Normally, SSCs are able to divide and differentiate to haploid germ cells (sperm) and maintain fertility in men during their life [19].

In 1994 the feasibility of isolating and transplanting SSCs to restore fertility in mice testes was established [20]. Since that time, several groups have tried successfully to transplant SSCs in other species including nonhuman primates [21]. This has given great hope in finding an effective alternative to preserve fertility in adult males, which may also represent a solution for restoring fertility in prepubescent boys following cancer treatment (Fig. 48.1). In addition to cancer survivors, other patients including but not limited to cryptorchidism (at risk of infertility because of undescended testes at birth) and Klinefelter syndrome (47XXY; at risk of testis fibrosis) may benefit from SSC implantation [19].

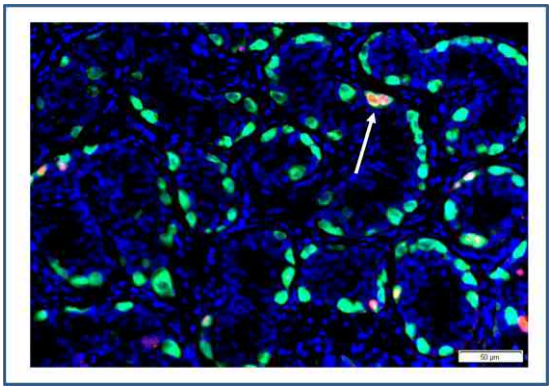

FIGURE 48.1 Spermatogonial cells (green fluorescent) in the base membrane of seminiferous tubules of a 5-year old boy. The cells were stained by MAGE-A4 antibody as a spermatogonia marker. Some of the spermatogonial cells have already started proliferation (*white arrow*; stained by Ki67 antibody). 4′,6-diamidino-2-phenylindole (DAPI) (blue) identified the nuclei of the cells. Scale bar 50 μm.

Nagano et al. [22] isolated human SSCs from six infertile men, which were transplanted to busulfan-treated nude mice. It was shown that human SSCs migrated into the base membrane of mouse seminiferous tubules of testis, but their numbers decreased significantly 1 month after transplantation. However, some SSCs remained in the mouse testis for up to 6 months without any evidence of differentiation [22]. Recently, a xenotransplantation study of prepubescent human SSCs showed similar results to the previous study on adult human SSCs [23]. The feasibility of SSC transplantation in a nonhuman primate model was demonstrated by Hermann et al. [21]. In this study, ejaculatory lentiviral positive sperm was found in the testes of more than 75% of adult (9 out of 12) and 60% of matured prepubescent (2 out of 5) recipient macaques monkeys for up to 63 weeks (average of 40.1 ± 4.9 weeks) after lentivirus-marked SSC transplantation. Autologous transplantation was twice as successful (70.5%; 12 out of 17) than allotransplantation (33.3%; 2 out of 6) in terms of presence of sperm in the ejaculate [21]. The ability of these sperms to fertilize eggs and develop preimplantation embryos was confirmed by performing ICSI. However, full pregnancies and live births were not observed in this study [21]. These results suggest that this technique could be translated to a clinical application in humans. In the previous study, an average of 88 ± 17.1 and 45.8 ± 14.5 million viable cells were used for transplantation to adult and juvenile testes, respectively [21]. It is well known that increasing the number of cells corresponds linearly with SSC transplantation success rate [24]. The number of SSCs in testis is very low (0.03% of germ cells and 1.25% of spermatogonial cells in mice; unknown in human) [25,26], and the size of testicular biopsy from premature boys undergoing cancer treatment is also very small (around 100 mm^3; containing 30,000 spermatogonia/mm^3) [19,27]. Although human SSC autotransplantation has not been tried yet, it is expected that successful human SSC autotransplantation requires in vitro expansion of these cells prior to transplantation. This culture method had been developed in animal models [26,28–30]. Recently, in a study involving the isolation and culture of human testicular cells from six different adult men who underwent orchiectomy as part of their prostate cancer treatment, we were able to maintain and propagate human SSCs in vitro for longer than 20 weeks [31]. The number of SSC increased more than 18,000-fold over 64 days of in vitro culture. In a follow-up study using testicular tissue from two boys aged 6.5 and 8 years, who were diagnosed with Hodgkin's lymphoma, isolated SSCs were able to propagate in vitro for at least 15.5–20 weeks [32]. Other groups have been successful in culturing human SSCs

[33–36]; however, this culture system should be optimized under good manufacturing practice regulations.

Before the first clinical trial of SSC autotransplantation in human, several safety issues must be addressed. First, especially in nonsolid cancer survivors such as hematopoietic malignancies, the transplanted cells should be free of any malignant cell contamination that may reintroduce the cancer to the recipient. In a study on culturing acute lymphoblastic leukemia (ALL) cells in combination with testicular cells from three different patients, ALL cells were undetectable beyond 26 days of culture even at extremely high initial concentration (40% ALL cells) [37]. However, it will be beneficial to test more cell lines from different types of leukemia to ensure complete purging of malignant cells. Second, the genetic integrity of stored, isolated, and propagated SSCs is very important, as it can influence the next generation of offspring. It has been shown that in vitro expanded mouse SSCs showed normal karyotype and stable androgenetic imprinting over 24 months [38]. Our work confirmed the genetic stability of in vitro propagated human SSCs; however, we found some changes in the epigenetic status of human SSCs during culture [39] .

Although trying for natural conception after SSC autotransplantation is ideal, testis tissue grafting or in vitro spermatogenesis followed by ICSI are other potential options to preserve fertility in many patients. Testicular tissue grafting and xenografting are extensively tested technologies that have been replicated in numerous mammalian species [40]. Immature testicular tissues from mice, pigs, goats, rabbits, hamsters, dogs, cats, horses, cattle, and monkeys have been grafted under the back skin of immune-deficient nude mice and matured to enable spermatogenesis, produce fertilization-competent sperm (pigs, goats, and monkeys) [41–43], and generate live offspring (mice, pigs, and monkeys) [43–46]. Therefore it is theoretically possible to graft immature testicular tissue from a childhood cancer survivor into an animal host to produce sperm that can be used in the in vitro fertilization (IVF) clinic to achieve pregnancy. However, the possibility that viruses or other xenobiotics could be transmitted from the animal host to humans needs to be carefully considered [47,48]. Recently, a model of prepubertal cancer survivor in rhesus macaques was introduced and reported that autologously grafted, frozen, and thawed prepubertal rhesus testicular tissues were matured to produce sperm that were competent to fertilize rhesus oocytes, establish a pregnancy, and produce a healthy graft-derived baby [49]. Complete spermatogenesis from grafted human tissues has not yet been achieved. Human tissue studies are needed to understand the scope, safety, and feasibility of testicular tissue grafting in patients. During the last three decades, in vitro spermatogenesis models have moved forward from initially conserving anatomical structure of the testis in culture to complete meiosis from testicular mouse germ cells [50]. Recently, we developed a three-dimensional (3D) testis organoid system from adult human testicular cells [47]. These human testis organoids (HTO) consist of SSCs, Sertoli, Leydig, and peritubular cells (Fig. 48.2A). Each organoid is approximately 300 μm in diameter and remain viable for at least 3 weeks (Fig. 48.2B and C) in an enriched medium with testicular ECM. This organoid system mimics in vivo spermatogenesis by supporting the differentiation of SSC to postmeiotic germ cells, albeit at a low frequency of approximately 0.2% [51]. The feasibility of using this system should be tested on prepubertal human testicular cells, and then the fertilization potential of the differentiated haploid cells should be analyzed as well.

Androgen-replacement therapy

Testosterone is the primary male sex hormone that plays multiple roles in the body. It has key roles in the development of male reproductive tissues such as testes and prostate, as well as promoting secondary sexual characteristics such as increased muscle and bone mass, and the growth of body hair [52]. Patients with testicular dysfunction and hypogonadal disorders may need androgen-replacement therapy to restore and maintain physiological levels of serum testosterone and its metabolites. Therefore testosterone therapy can increase muscle strength, stabilize bone density, improve osteoporosis, and restore secondary sexual characteristics, including libido and erectile function [53]. However, long-term exogenous testosterone therapy is not optimal and can cause multiple problems, including excessive erythropoiesis, bone density changes, and even infertility [54]. To address these problems, researchers are looking for an effective method to provide sufficient endogenous testosterone. In an animal model, isolated Leydig cells from rats that were cultured and stimulated with human chorionic gonadotropin (hCG) showed a high level of testosterone production [55]. In this study, engineered alginate-poly-L-lysine-encapsulated Leydig cell microspheres (with an average diameter of 0.7 ± 0.06 mm) were injected into castrated rats. Approximately 10% of a normal adult rat Leydig cell population was injected into each castrated animal. The serum testosterone levels increased and were maintained up to 40% of normal for a maximum period of 43 days, without any HCG stimulation [55]. Bilateral testicular agenesis or atrophy often requires the placement of testicular prostheses and androgen supplementation [56]. In this regard the possibility of creating hormone releasing testicular prostheses has been investigated [57]. To form testicular prosthesis, isolated chondrocytes from bovine articular cartilage were seeded on testicular shaped

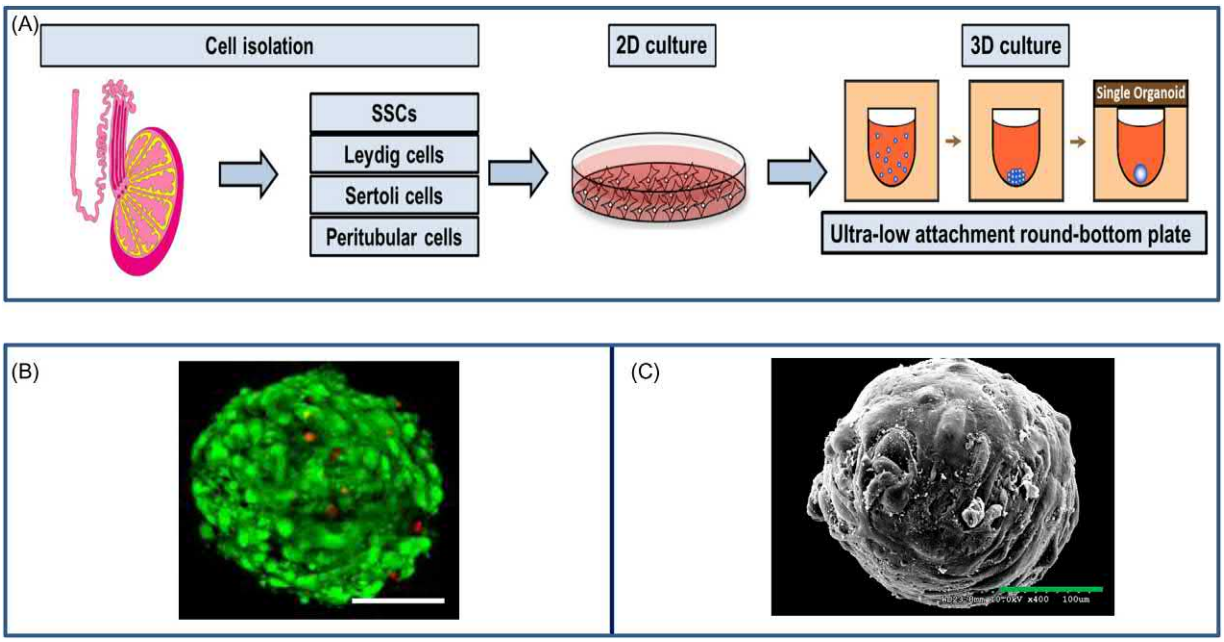

FIGURE 48.2 Human 3D Testis organoid system (HTO). (A) Testicular cells, including SSCs, Leydig cells, Sertoli cells, and peritubular cells, are isolated and propagated in 2D culture, then seeded into ultralow attachment round-bottom plates for 3D formation of organoids. The HTO formation takes 48–72 h. (B) Live/dead staining of organoid revealed maintenance of viability throughout the culture period. Green and red fluorescents represent alive and dead cells, respectively. (C) Ultrastructural analysis of multicellular human testicular organoids using SEM to show exterior morphology. Scale bars are 100 μm. *3D*, Three-dimensional; *SEM*, scanning electron microscopy; *SSCs*, spermatogonial stem cells.

polymer scaffolds. Implanted engineered testis prostheses loaded with 100 μg testosterone enanthate were able to maintain the physiologic levels of testosterone in vivo for at least 16 weeks in recipient mice [57]. For a better outcome, combinations of Leydig cell technology and engineered testicular prosthesis need to be further explored.

As mentioned earlier, we have recently developed a 3D testis organoid system from adult human testicular cells (HTOs) [51]. This HTO system consists of SSCs, Sertoli, Leydig, and peritubular cells. The Leydig cells in this multicellular structure could produce androgen with and without hCG stimulation [51] (Fig. 48.3).

Future work should focus on the feasibility of implanting adequate numbers of these HTO to castrated animal models as a potential, novel androgen-replacement therapy.

Ejaculatory system

Ejaculation is the process of transporting sperm through the urethra and expelling semen from the urethral meatus. The organs involved in the ejaculatory process are the epididymis, vas deferens, prostate, seminal vesicles, bladder neck, external urethral sphincter, and bulbourethral glands. In normal ejaculation, anatomic structures are precisely coordinated via neural centers to allow normal ejaculation to take place [58,59].

Engineering vas deferens

Congenital bilateral absence of the vas deferens (CBAVD) causes obstructive azoospermia which is common in about 1% of infertile men [60]. As CBAVD men have normal spermatogenesis, they are able to achieve biological fatherhood with the help of assisted reproduction techniques, that is, surgical sperm extraction, and IVF [61]. In addition, conception also requires the female partner of CBAVD men to undergo ovarian stimulation for IVF/ICSI procedure. A solution to this problem would be replacing the vas deferens. A novel approach for the tissue engineering of vas deferens has been tried in a rabbit model [62]. Polyethylene tubes (with 0.86 mm diameter and 6 cm length) were implanted into the peritoneal cavities (as an in vivo bioreactor) of 16 rabbits [62]. After 2–3 weeks the tubes were removed and the encapsulating myofibroblast-rich tissue that resulted from the foreign body response to the biomaterial was harvested for grafting as vas deferens in the rabbits [62]. At 2, 4, 6, and 8 months postimplantation, morphological and immunohistochemical evaluations showed that the structure of engineered vas deferens was similar to native vas deferens tissue [62]. In addition, the presence of sperm in the ejaculate indicated normal function of the engineered vas deferens. However, more research on large animal models is required before this approach can be used in humans.

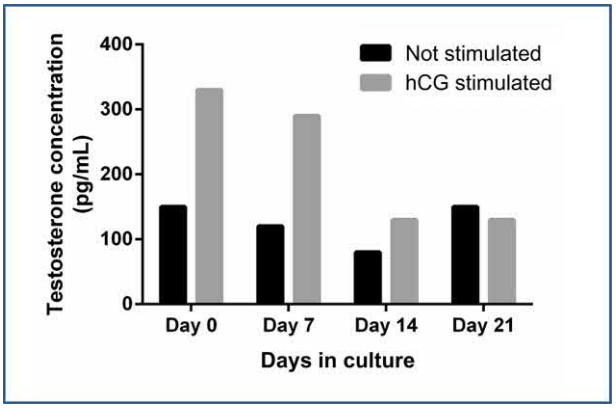

FIGURE 48.3 Androgen production by Human 3D Testis organoid system (HTO). Testosterone concentration in organoid culture media was measured over 3 weeks. Organoids were treated with hCG to stimulate testosterone production. Media from organoids treated with hCG was compared to control media from organoids with no hCG treatment. On day 0, 7, and 14, organoids produced more testosterone when stimulated with hCG. However, on day 21, organoids did not produce more testosterone when stimulated with hCG. The lack of increase in testosterone production on day 21 in response to hCG stimulation could be due to organoids becoming desensitized to hCG (in each time point the secreted hormone from a group of 16 organoids were pooled; $n = 16$). *3D*, Three-dimensional; *hCG*, human chorionic gonadotropin.

Spinal ejaculation generator

The incidence of anejaculation in the population is small, but when present, it represents a formidable challenge to the infertile couple and their treating physician [63]. Spinal cord injury (SCI) is the most common cause of anejaculation encountered in clinical practice [64]. The incidence of traumatic SCI is estimated at 16 individuals per million per year in Western Europe and 39 individuals per million per year in North America with a prevalence of 300 and 853 individuals per million, respectively [65]. In the United States the mean age at occurrence of SCI is 37.1 years, and 77.1% of SCI patients are male [66]. Ejaculation can be physiologically defined as the rhythmic, forceful expulsion of semen at the urethral meatus. Ejaculation comprises two successive phases, emission and expulsion, each involving different pelvic–perineal anatomical structures [67]. Emission is controlled by autonomic (sympathetic and parasympathetic) spinal centers, and expulsion is controlled by somatic spinal centers. These centers act in synchrony for antegrade ejaculation to occur. Such synchronization has been reported to be led, in rats, by a group of lumbar spinothalamic neurons forming the spinal generator of ejaculation (SGE). The critical role of the SGE and its organization have been described during the last decade in functional and neuroanatomical studies [67–71]. Penile vibratory stimulation (PVS) is a procedure that uses external vibration to stimulate the sensory nerves on the penis, with resultant reflex ejaculation (Fig. 48.4A). Electro ejaculation is an option for SCI men with lower motor neuron lesions and for those who have failed PVS, but it is somewhat more invasive. This technique uses electricity delivered via a rectal probe to recruit the ejaculatory reflex. The Seager Electroejaculator (Dalzell Medical Systems, The Plains, VA) remains the only FDA-approved device for this procedure (Fig. 48.4B).

In the past two decades numerous studies have focused on therapeutic approaches of SCI in animal models. Significant progress on functional remyelination [72], direct injection of biological agents [73], formation of neuronal relays [74], biomaterial approaches to enhancing neurorestoration [75], and neural stem cells transplantation [76,77] have been made. These studies promise translation to more clinical trials on SCI and neuronal regeneration. Although the main aim of these studies is correcting motor and sensory deficits, it will be interesting to evaluate the improvement on ejaculation function of these patients too.

Penis

Penile reconstruction

Congenital and acquired genitourinary tract anomalies of the penis require surgical reconstruction. Lack of sufficient normal autologous tissue is the major limitation in many surgical procedures for different conditions such as ambiguous genitalia, epispadias, hypospadias, micropenis, aphallia, severe chordee, impotence, female-to-male genital reassignment, and traumatic or iatrogenic penile defects [78–80]. The possibility of developing tissue composed of corporal cells in vivo has also been investigated [81]. Human corporal smooth muscle cells (SMC) and endothelial cells (EC) were seeded on biodegradable polymer scaffolds at concentrations of 20×10^6 and 10×10^6 cells/cm^3, respectively. These constructs were then implanted in the subcutaneous space of nude mice [81]. Histological evaluation showed the formation of vascularized corpus cavernosum comprising these two cell types 28 and 42 days postimplantation [81]. To achieve a 3D corporal structure, naturally derived polymer scaffold from rabbit corporal tissue was used in combination with primary human cavernosal SMC and EC [82]. Engineered corporal tissues were maintained in culture for up to 4 weeks and then implanted into nude mice [82]. The matrices showed a stable collagen concentration 8 weeks postimplantation [82]. Immunocytochemical studies using α-actin and factor VIII antibodies confirmed the presence of corporal SMC and EC in vivo [82]. Organ bath studies also showed that the cell-seeded corporal tissue matrices responded to electrical field stimulation [82]. In a follow-up study, structural parameters were evaluated up to 6 months postimplantation in a rabbit penile defect model

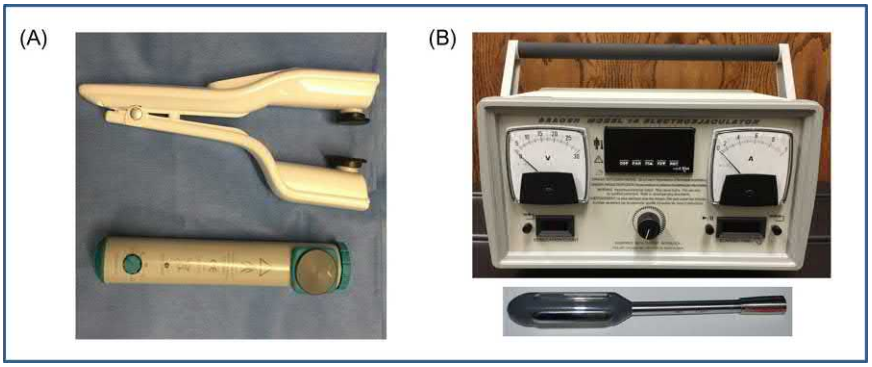

FIGURE 48.4 Currently available instruments to collect semen from spinal cord injury patients. (A) PVS including FertiCare (bottom device) and Viberect X3 (top device). These devices can be placed on either the dorsum or the frenulum of the penis (or both). (B) Seager EEJ and its transrectal probe. The probe is used to deliver electrical current directly to the prostate and seminal vesicles, resulting in ejaculation. The harvested sperm can be used for either IUI or IVF. *EEJ*, Electro ejaculation; *IUI*, intrauterine insemination; *IVF*, in vitro fertilization; *PVS*, penile vibratory stimulators.

[83], where the engineered corporal bodies showed structural integrity on cavernosography. The presence of sperm after copulation was confirmed in rabbits with the engineered corpora [83]. In all of these studies, it was shown that a short segment of the penile corporal body (approximately one-third of the penile corpora) can be replaced using naturally derived collagen matrices with autologous cells [81–83]. To improve the results of previous studies, engineering of the entire length of both penile corpora was attempted [84]. In another study the matrices were seeded with the autologous corporal SMC and EC, using a novel multistep cell seeding protocol [85], and the cell-seeded matrices were used to replace the entire pendular penile corpora in 12 male rabbits [84]. At 1, 3, and 6 months following the implantation of the engineered corpora, the rate of sperm presentation on vaginal swabs and pregnancy rates of mated female rabbits were found to be 83% and 30%, respectively. Untreated rabbits showed no evidence of intravaginal sperm and no pregnancies happened [84]. These studies demonstrate that penile corpora cavernosa tissue can be engineered and transplanted to restore function.

Penile transplantation

As an option for penile reconstruction, penile transplantation has been considered, especially after near-complete penile loss. So far, three allogenic penile transplantations have been performed worldwide. The first one was performed in 2006 in China successfully; however, it was removed after 2 weeks because of severe psychological problems of the recipient and his wife [86,87]. The second case was described in 2014 and performed in South Africa. The surgery was complicated by a reintervention after 4 days to remove a thrombus from the anastomosis and another reintervention for hematoma drainage. The patient, however, reported normal sexual and urinary function 3.5 months after transplantation [88]. The third case was the first US-based penile transplantation and performed in a patient suffering from penile cancer [89]. The requirement of lifelong, multidrug immunosuppression bearing the risk of serious side effects still remains a limiting factor for widespread clinical application of penile transplantation [90].

Stem cell therapy for erectile dysfunction

ED is defined as the consistent or recurrent inability to achieve or maintain an erection sufficient for sexual activity [91]. The interest on recruiting either endogenous or exogenous stem cells to treat ED is increasing [92]. It is generally believed that tissue-specific stem cells exist in most postnatal tissues. So far, two types of foreskin stem cells have been isolated to date, including skin-derived progenitors [93] and MSCs [94]. In a rat study using EDU labeling the presence of stem/progenitor cells has been shown. These cells, mainly distributed within the subtunic and perisinusoidal space of penis, are defined as subtunic penile progenitor cells and perisinusoidal penile progenitor cells. These cells expressed c-kit, A2B5, and PCNA [95]. It is expected that reactivation of endogenous stem cell potential might help the rejuvenation of damaged erectile function [96]. Human MSCs have been isolated from a large number of adult tissues (bone marrow, adipose tissue, skeletal muscle, etc.). Adipose tissue, being a rich and easily obtainable source of MSC, has been of particular interest in the treatment of ED [97]. MSCs express low levels of MHC class I and do not express MHC class II molecules; thus they are minimally immunogenic. Therefore they can be used from either autologous or allogenic sources [98]. Isolated MSCs from rat paratesticular fat tissues were injected in a bilateral cavernous nerve injury model. One month after injection, measurement of intracavernous pressure revealed significant improvement on ED [99]. Effect and mechanisms of low-energy shock wave (LESW) therapy has been tested in a rat ED model induced by pelvic neurovascular injuries. After 4 weeks of LESW treatment and 2 week of wash out, all rats underwent erectile function measurement. LESW treatment improves erectile function by

leading to angiogenesis, tissue restoration, and nerve generation with more endogenous EdU + progenitor cells recruited to the damaged area and activation of Schwann cells. LESW facilitates more complete reinnervation of penile tissue with regeneration of neuronal nitric oxide synthase−positive nerves from the major pelvic ganglion to the penis. In vitro treatment of Schwann cells with ESWL demonstrated its direct effect on cell proliferation. Western blot analyses showed that Schwann cell activation−related markers including p-Erk1/2 and p75 were upregulated after LESW treatment [100]. In another rat injury model, long-term therapeutic effect amongst three different types of human cell on ED has been tested. Two and half million cells of umbilical vein EC (UVEC), adipose-derived stem cells (ADSC), or amniotic fluid−derived stem cells (AFSCs) were injected intracavernously into the penile tissue. Saline injection was served as a control group. Erectile function and histomorphological analyses of penile tissues were assessed 12 weeks after defect creation and cell or saline injection. The ratio of intracavernous pressure to mean arterial pressure (functional indicator) was significantly higher in the cell therapy groups compared to the saline-injected control group ($P < .05$). Immunofluorescence staining showed more cells expressing biomarkers of endothelial, smooth muscle, and nerve cells within the penile tissue in the cell therapy groups when compared to the control group. This study demonstrated the beneficial effects of AFSC, UVEC, and ADSC on the penile corporal sinusoids showing a recovery of about 80%, 70%, and 60% of normal erectile function, respectively [101]. All of these observations demonstrate how stem cell treatment is a promising tool to restore normal erectile function, but these applications need further development for future clinical trials.

Conclusion

Regenerative medicine is a branch of translational research in tissue engineering and molecular biology, which deals with the process of replacing, engineering, or regenerating human cells, tissues, or organs to restore or establish normal function of tissues and organs. In the past two decades, most of the effort toward engineering of male reproductive tissues has occurred in animal models. Safety of engineered reproductive tissues is usually more critical than other organs because, in many cases, more than just recipients are involved. The health of offspring as well as the patient's partner should be considered carefully in any clinical trials in the future. Regenerative medicine has opened new avenues to treat disorders of male reproductive system affecting fertility and sexual life.

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Part Fourteen

Musculoskeletal system

Chapter 49

Mesenchymal stem cells in musculoskeletal tissue engineering

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Introduction

A half century ago, Friedenstein et al. [1] described a population of nonhematopoietic cells isolated from human bone marrow with the in vitro ability to adhere, proliferate, and differentiate into chondrocytes, osteoblasts, and adipocytes. Such cells were identified in small amounts in multiple tissues throughout the body. Because of their differentiation properties and the ease of their isolation and in vitro expansion, these mesenchymal stem cells (MSCs) were considered an attractive candidate progenitor cell type for tissue engineering and regenerative medicine applications. Extensive investigation has yielded knowledge about their biology, particularly tools for controlling their activity and cell fate that are critical to their clinical use. Beyond their original identification in bone marrow–derived cells, MSC-like cells have been shown to have more tissues of origin, as well as differentiation capabilities that include various cells of mesenchymal lineages, including cellular phenotypes representative of the musculoskeletal tissues, such as cartilage, bone, muscle, ligament, and tendon. A plethora of factors control the extensive proliferative and multipotent differentiation capacity of MSCs, including cytokines, hormones, various soluble and insoluble signaling molecules, and transcription factors. The development and maturation of musculoskeletal tissues demand specific form and mechanical strength, and the success of a tissue-engineered outcome depends on the dynamic interactions between scaffolds, cells, and various physical and chemical environmental cues. By exploiting the current knowledge of MSC, promising and exciting results have been generated toward engineering tissue replacements for cartilage, bone, osteochondral, and other musculoskeletal tissues. Future clinical successes will rely on significant knowledge-based improvements. The current understanding, recent advances, and remaining challenges for the deployment of MSCs for musculoskeletal tissue-engineering applications constitute the subject of this chapter.

Mesenchymal stem cell biology relevant to musculoskeletal tissue engineering

An ideal cell source for tissue engineering should exhibit the following characteristics: easy access and high availability of the source cells, capacity for extensive self-renewal or expansion to generate sufficient quantity, the capacity to differentiate readily into cell lineages of interest upon instructive differentiation cues, and/or the ability to modulate the native environment to promote appropriate tissue repair and integration, including minimal immunogenic or tumorigenic ability. We will discuss below the intrinsic properties of MSC that determine their behavior, as well as external factors that affect the growth, differentiation, and the developmental outcomes of MSCs.

Mesenchymal stem cell identification

Since their initial description in the 1960s [1], MSCs have been shown to possess the capacity to differentiate into cells characteristic of several mesenchyme-derived tissues, including cartilage, bone, fat, muscle, tendon, and hematopoietic-supporting marrow stroma (reviewed in Ref. [2–7]). In addition to these mesenchymal lineages, MSCs were shown to differentiate into other cell/tissue types, including hepatocyte-like cells [8] and neural-like tissues [9,10]. However, the functionality of these nonmesodermal MSC-derived cells remains debatable.

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Experimentally, MSCs are identified and isolated first by their ability to adhere to plastic. Next, proliferative and differentiable MSCs are identified, at a minimum, by the expression of a combination of surface markers [11]. These include STRO-1, SB-10, 5′-nucleotidase (CD73), and SH4 antigens as well as Thy-1 (CD90), TGF-β receptor type III endoglin (CD105), hyaluronic acid receptor CD44, integrin subunits α1 (CD49a), α2 (CD49b), α5 (CD49e), αV (CD51), β1 (CD29), activated leukocyte-cell adhesion molecules (CD166), melanoma cell adhesion molecule (CD146) [12,13], cell surface peptidase CD13 [14], and possibly others [2,15]. MSCs are negative for the hematopoietic markers, CD19/CD79a, CD34, CD45, CD11b/CD14, and HLA-DR [2]. CD73, SH4, and STRO-1 antibodies recognize antigens that are present on MSCs and other cells but not hematopoietic cells (for review, see Refs. [16–18]). The presence of CD75, CD90, and CD105 and absence of hematopoietic markers, while not sufficient to fully identify an MSC, has been suggested by the International Society of Cell Therapy as the minimum positive criteria necessary to consider a potential cell an MSC [19]. It therefore remains a challenge to isolate MSCs specifically from a mixed cell population. Frequently, a combination of antibodies is used to retrospectively characterize the MSC phenotype. Inconsistency in the literature on the growth characteristics and differentiation potential of MSCs underscores the need for a clear functional definition of MSCs [20]; at present the sole characteristic uniting disparate MSC sources is their ability to

1. adhere to tissue culture plastic under standard culture conditions;
2. form colonies, that is, a single cell giving rise to a colony of undifferentiated cells;
3. proliferate, that is, to self-renew and remain in an undifferentiated state until provided the signal to differentiate; and
4. differentiate along specific mesenchymal lineages when induced (Fig. 49.1).

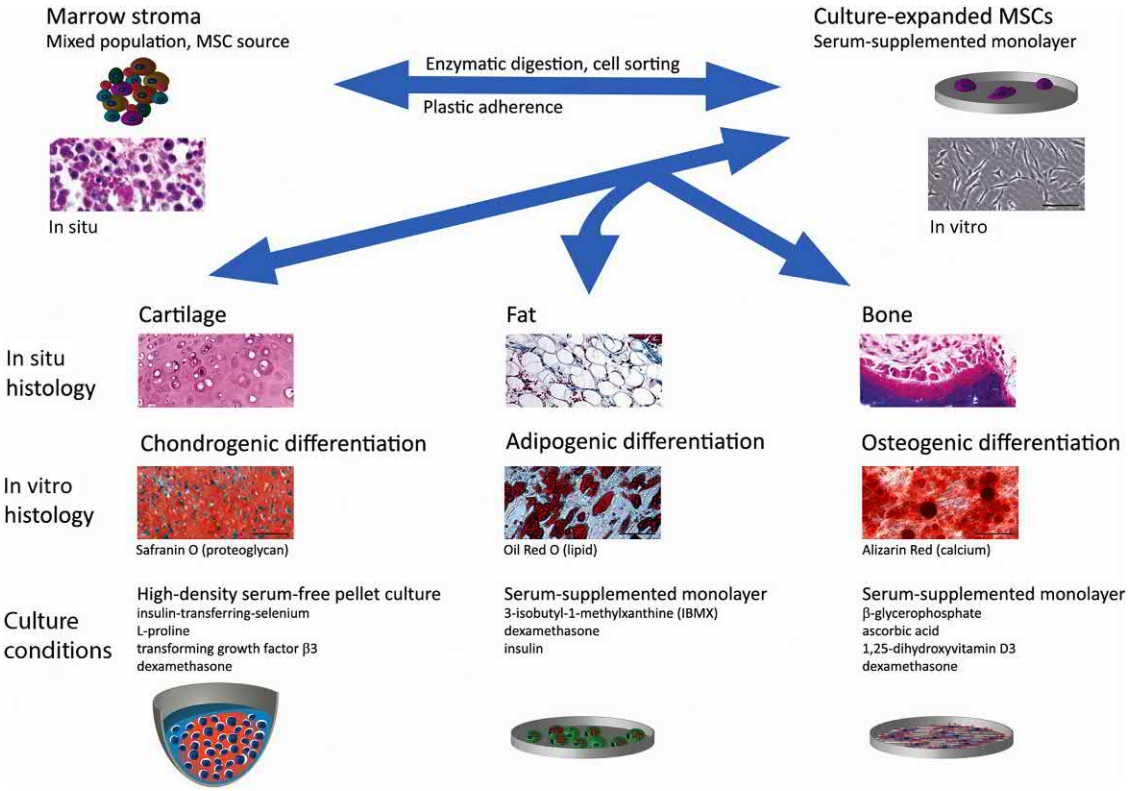

FIGURE 49.1 Minimum multilineage differentiation potential of mesenchymal stem cells. MSCs are able to differentiate into cell types of multiple lineages under appropriate conditions (adapted from [21]). Classically, these lineages include but are not limited to bone, cartilage, and fat. Bone marrow–derived MSCs, specifically, can repopulate the bone marrow stroma under certain conditions. Strong evidence exists that MSCs, once differentiated in vitro, can be dedifferentiated and subsequently used without harm to their multipotency. In addition to the standard lineages shown previously, additional work suggests that this transdifferentiation ability also applies to in vitro tenogenesis and musculogenesis (adapted from [22]). In situ and in vitro histology is shown for phenotype comparison (scale bar = 100 μm). *MSCs*, Mesenchymal stem cells. *Adapted with permission from Tuan RS, Boland G, Tuli R. Adult mesenchymal stem cells and cell-based tissue engineering. Arthritis Res Ther 2003;5(1):32–45.*

Tissue sources of mesenchymal stem cells

While bone marrow remains the best studied tissue source for MSCs, MSC-like cells have been identified in a number of other tissues. That MSCs or MSC-like cells can be found in almost all organs that harbor connective tissues implies that these cells may have a fundamental functional role in the body. The endogenous function(s) and exact location of these stem cells in the various tissues are under investigation, and they are generally thought to participate in local tissue repair and regeneration. Bone marrow MSCs can give rise to stromal cells that support hematopoiesis in vitro and in vivo, possibly providing extracellular components as well as various growth factors and cytokines [23]. Besides the bone marrow, other tissues of origin of MSCs have been investigated and their relevance to MSC biology debated. In 2008 Crisan et al. demonstrated that, independent of tissue sources, pericytes, which are mural cells located in direct contact with endothelial cells, while embedded within the basement membrane of small blood vessels, are indeed MSCs [12]. From this and other following studies, it was concluded that the perivascular niche serves as a universal source of MSCs, which are generally thought to participate in local tissue repair and regeneration [24]. Nevertheless, MSC-like cells could also be isolated from avascular (or less vascular) tissues such as human adult articular cartilage [13] and tendon [25,26].

Due to their early identification, MSCs from bone marrow have been widely investigated for regenerative therapies. Bone marrow is relatively easy to access, but isolation of bone marrow is an invasive procedure accompanied by potential donor site morbidity, pain, and infection. In addition, the low cell incidence of 0.001%–0.01% of the overall cell population and a relatively long cell doubling time (approximately 60 hours) [5,27] have prompted researchers to find alternative cell sources for MSC isolation [16–18,28,29] (Table 49.1). Adipose tissue was found to be an abundant source of MSCs, which yields 500 times more MSCs than an equal amount (volume) of bone marrow [67]; in addition, adipose-derived cells exhibit a high proliferation potential with a doubling time of approximately 20 hours [68,69] (Table 49.2). Other easily accessible sources of MSCs include a number of perinatal tissues, such as placenta, umbilical cord and umbilical cord blood [70], amnion [71], as well as urine [72,73] and the more recently emerging endometrial MSCs [74], which can be derived from menstrual blood [75,76].

TABLE 49.1 Tissue sources of human mesenchymal stem cells.

Tissue source	Representative references
Bone marrow	[1,5,6]
Trabecular bone	[30,31]
Muscle	[32–34]
Adipose	[35,36]
Periosteum	[37,38]
Synovial membrane	[39,40]
Articular cartilage	[13,41–43]
Skin	[32,44]
Pericyte	[12,45]
Peripheral blood	[46,47]
Deciduous teeth	[48]
Periodontal ligament	[49,50]
Palatine tonsil	[51,52]

In addition to bone marrow and the above tissue sources, human MSCs have been identified in and isolated from intestinal [53], limbal stroma of eyes [54], knee-joint tissues [55–58], prostate stroma [59], trachea [60], nasal mucosa [61], WJ [62,63], umbilicus and cord blood [64,65], and placenta [66]. *MSCs*, Mesenchymal stem cells; *WJ*, Wharton's jelly.

TABLE 49.2 Culture medium formulation and growth characteristics of human bone marrow–derived mesenchymal stem cell.

Expansion culture media	Population doublings	References
αMEM, 17% FBS, 2 mM L-glutamine	10	[77]
Coon's modified Ham's F12 medium, 10% FCS	22–23	[78]
αMEM, 10% FBS	15–20	[79]
αMEM, 10% FCS	24 ± 11 to 41 ± 10	[27]
αMEM, 20% FBS, 100 μM L-ascorbate-2-phosphate	20	[80]
Coon's Modified Ham's F-12, 10% FCS, 1 ng/mL FGF-2	22–23	[81]
DMEM, 10% FBS	38 ± 4	[82]
αMEM, 20% FBS	30	[83]
DMEM, 2% FBS, MCDB-201, 10 ng/mL EGF, 1 × insulin–transferrin–selenium, 1 × linoleic acid, BSA, 10 nM dexamethasone, 10 mM ascorbic acid phosphate	30	[84]

DMEM, Dulbecco's Modified Eagles Medium; *EGF*, epidermal growth factors; *FGF*, fibroblast growth factor; *αMEM*, α-Minimum Essential Medium; *FCS*, fetal calf serum; *FBS*, fetal bovine serum; *BSA*, bovine serum albumin.

However, it is noteworthy that the multilineage differentiation and regeneration potencies of MSCs or progenitor cells derived from different tissues are still under investigation and debate. MSC yield, proliferation rate, multipotency, differentiation potential, and the previously mentioned accessibility determine the usefulness of MSCs from different tissue origins. Although MSCs derived from different tissues share a number of phenotypic characteristics, they clearly are not identical, as they show different propensities in proliferation and differentiation. For example, a study comparing human MSCs derived from bone marrow, umbilical cord blood, and adipose tissue showed that adipose tissue contained the highest number of MSCs, while umbilical cord blood contained the lowest; however, umbilical cord blood–derived MSC could be cultured for the longest period of time and showed the highest proliferation capacity, whereas bone marrow–derived MSC showed the lowest proliferation capacity [68]. Sakaguchi et al. compared the properties of MSCs isolated from bone marrow, synovium, periosteum, skeletal muscle and adipose tissue, and found significant differences in their multilineage differentiation potential [85]. MSCs from bone marrow, synovium, and periosteum showed greater chondrogenic activities, with synovium-derived MSCs exhibiting the highest capacity. Adipose-derived MSCs exhibit enhanced chondrogenesis if expanded in the presence of excess fibroblast growth factor (FGF)-2, then chondrogenically induced with bone morphogenetic protein (BMP)-6 (BMP6) [86,87]. With regard to osteogenesis the rate of matrix mineralization was highest in bone marrow, synovium, and periosteum-derived MSCs. Therefore the optimal source of MSCs for therapeutic use for the musculoskeletal system remains to be determined.

In addition to tissue source, donor age, life style, and disease stage may also directly affect MSC yield, rate of proliferation, multipotency, and regenerative potential. Decreasing MSC number and proliferation rate as well as differentiation potential were observed with increasing donor age [88]. MSCs from obese donors had a significantly impaired proliferation and differentiation potential [89]. When isolated from osteoporotic women, MSCs showed significantly reduced proliferative response and osteogenic differentiation [90]. With bone marrow MSCs, advanced osteoarthritis condition, regardless of age, had a significantly deleterious impact on proliferative capacity and chondrogenic and adipogenic activities when compared to healthy donors [91]. Decreases in MSC quantity and quality due to age and disease may limit the use of autologous MSCs in clinical settings, warranting further study of allogeneic MSCs for tissue repair. In addition to disease transmission, these nonautologous tissues might carry the risk of immune rejection and allogeneic reaction by the host and will be discussed in detail later.

Mesenchymal stem cell isolation and in vitro culture

Some adult tissues, such as adipose tissue, contain stem/progenitor cells with similar characteristics of MSCs and have higher occurrence rate (1%–5% in lipoaspirates) than in bone marrow, and the exact number depends on the isolation and characterization methods used [92]. Various protocols of MSC isolation are available, and some are species specific.

It is important to note that the isolation method of MSCs also impacts the number as well as quality of isolated cells. Depending on the tissue source, a number of enzymatic, mechanical, explant culture, and density-gradient centrifugation methods are employed (reviewed in Ref. [93]). To extract MSCs from solid tissues, proteolytic enzymes, for example, collagenase, dispase or trypsin, are used. These are sometimes coupled with mechanical dissociation methods, such as shear force and centrifugal force, especially for the isolation of MSCs from adipose tissue. A less frequently used approach is explant culture, where cells are allowed to grow out from small tissue pieces, for example, in the isolation of trabecular bone-derived MSCs [30,94].

Human bone marrow MSCs can be isolated by simple direct plating after isolation from native tissue, sometimes involving enzymatic digestion [95]. Frequently, this is performed after density-gradient centrifugation over Ficoll or Percoll, where a layer of mononuclear cells containing MSCs is obtained. With culture time and medium changes, nonadherent hematopoietic cells are washed away, and MSCs appear as small, adherent, spindle-shaped fibroblast-like cells.

As none of these methods allow immediate distinction between stem and nonstem cell populations, surface marker–based selection is often utilized to enrich MSCs in the isolated cells. These methods include positive or negative selection using either magnetic-activated cell sorting or fluorescence-activated cell sorting to further purify the MSC population. Positive selections have been based on the detection of markers that are expressed, albeit not exclusively, by MSCs; negative selection is based on the exclusion of markers that are expressed by hematopoietic cells, using antibodies against CD34, CD45, and CD11b, as mentioned previously.

Because of their low abundance in adult tissues, the number of MSCs in the primary isolate is often insufficient for clinical applications, necessitating the expansion of MSCs in vitro. Growth medium cocktails, designed to maintain the multipotent and proliferative capacities of MSCs, vary widely and typically involve culture in serum-supplemented Dulbecco's Modified Eagles Medium (DMEM) or α-Minimum Essential Medium (Table 49.1). Similar procedures are applicable for MSCs and progenitors derived from other tissues.

After initial expansion, cultures are maintained at densities of 50–100,000 cells/cm^2 [4,96,97]. The extensive expansion capacity of MSCs depends on the harvesting techniques, culture conditions, and the health condition and age of the donor. Culture conditions, such as initial seeding density and glucose levels in the medium, also influence the expansion capacity of MSCs. For example, higher proliferation profiles of MSCs was seen when plated at low initial plating density (1.5–3 cells/cm^2) but not at high density (12 cells/cm^2), resulting in a dramatic increase in the fold expansion of total cells (2000-fold vs 60-fold increase, respectively) [83]. Another example is the observation that tissue-specific progenitor cells grow faster in low glucose (5.5 mM), DMEM-based growth medium compared to high glucose (25 mM) culture medium, as the former condition better supports stem cell maintenance and prevention of cell aging [13,83,98].

Since the use of animal-derived serum increases the risk of transmission of zoonotic agents and the presence of xenogenic components in MSC products may elicit potential immunogenicity, the use of human-derived serum and platelet lysate has been explored [99,100]. Ideally, serum-free, chemically defined media are preferred and are being developed for isolation and expansion of MSCs under good manufacturing practice compliance [101]; in this manner, MSC expansion strategy may be readily customized for different applications [102].

Mesenchymal stem cell self-renewal and proliferation capacity

In vivo, MSCs likely remain in a mitotically quiescent state (G0 stage of the cell cycle) as demonstrated by the analysis of fresh bone marrow cell harvests continuously exposed to tritiated thymidine labeling [103]. BrdU labeling of cells in a human trabecular bone explant culture also revealed that cell proliferation, including that of STRO-1 positive mesenchymal stem/progenitor cells, was inhibited completely within the explant tissue milieu [104]. Since most of the tissues/organs from which MSCs are derived exhibit a relatively slow turnover rate in the adult organism, it is not unreasonable to speculate that self-renewal or proliferation of MSCs is normally suppressed in vivo in the course of tissue homeostasis, possibly regulated by intrinsic factors present in the tissue microenvironment and/or through direct or indirect interaction with neighboring cells. Once homeostasis is altered by injury or disease, the tissue progenitor cells or MSCs are activated, and cell behaviors, including proliferation and migration, can be observed, such as in the case of cartilage-derived stem/progenitor cells in an osteoarthritic articular cartilage (Fig. 49.2) [105,106].

In vitro cell cycle studies revealed that the majority of MSCs (between 78.7% and 96.45%) are in the G0/G1 phase of the cell cycle while a very small fraction of MSCs are engaged in active proliferation. During the exponential phase of cell growth, human MSCs isolated from bone marrow and fetal pancreas exhibited a similar population doubling time, ranging from 10 to 30 hours [107].

Unlike embryonic stem cells, MSCs do not expand indefinitely in vitro when cultured in the presence of serum, which could be a reflection of their intrinsic properties as adult stem cells or their exposure to a suboptimal culture environment. After extensive propagation, MSCs change their phenotype from a fibroblastic shape to a

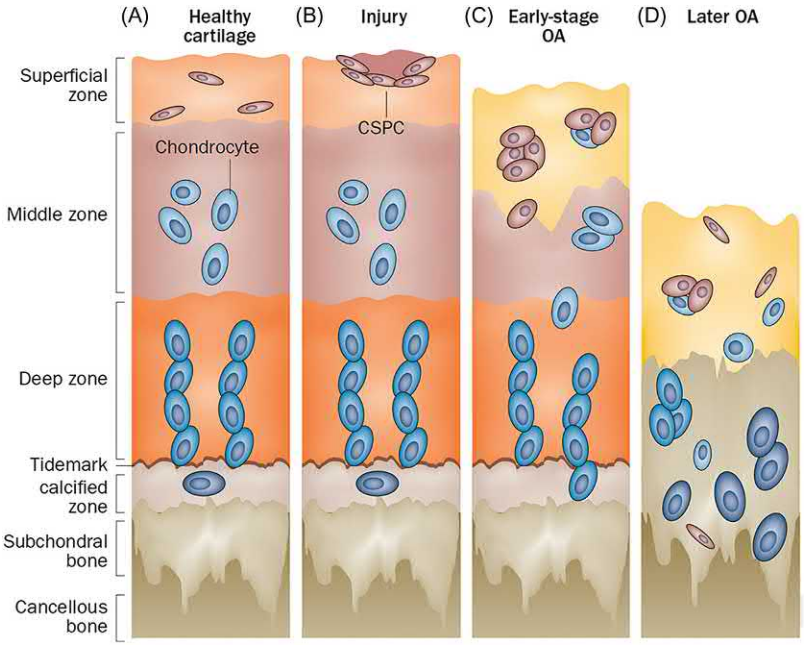

FIGURE 49.2 Human articular cartilage and schematic of CSPCs distribution during osteoarthritis pathogenesis. Zonal architecture and chondrocyte distribution in healthy adult articular cartilage, showing the superficial zone, middle zone, deep zone, tidemark, calcified zone, subchondral bone, and cancellous bone. (A) CSPCs (red) in normal adult cartilage—probably at superficial layer, and Notch-1 positive. (B) CSPC (red) response to injury—spindle shaped cells appear and migrate to the injury site. CSPCs in early OA (red)—with loss of superficial layer and changes in the internal structure of articular cartilage—stem cell markers are expressed and cell clusters emerge, containing cells positive for stem cell markers. (D) CSPCs in late stage OA (red)—with continuing matrix loss and chondrocyte hypertrophy, CSPCs appear to migrate throughout the articular cartilage. *CSPCs*, Cartilage-derived stem/progenitor cells. *Adapted with permission from Jiang Y, Tuan RS. Origin and function of cartilage stem/progenitor cells in osteoarthritis. Nat Rev Rheumatol 2015;11 (4):206–12.*

more flattened morphology with extensive pseudopodia and actin stress fiber formation, a phenomenon usually referred to as "replicative senescence." Consistent with their limited expansion capacity, human MSCs do not have high telomerase activity as compared to immortalized cells, and their telomere length is reduced as the cells grow older [108]. However, the telomerase activity appears to play a crucial role in maintaining the self-renewal and multidifferentiation potential of MSCs. Thus murine MSCs derived from telomerase knockout mice failed to differentiate into adipocytes and chondrocytes and lost telomere at late passage [109]. On the other hand, when transduced with telomerase, hMSCs stably expressing telomerase reverse transcriptase could undergo 80 [110,111] or 260 [112] population doublings without growth arrest.

Presence of growth factors and specific culture conditions also play a role in the expansion capacity of MSCs. Thus basic FGF (bFGF) can prolong the replicative capacity of hMSCs and increase the total cell numbers several-fold when included in the basal culture medium [113]. Other growth factors and cytokines, including canonical Wnts, for example, Wnt 3A [114], and interleukin-6 (IL-6) [115], have also been found to enhance the proliferative activity of adult human MSCs. Growth factor pretreatment may also help define the differentiation potential of the cultured MSCs [116], and this type of primed-MSCs are more ready to reenter the differentiation state of primed-lineages compared to unprimed MSCs [117,118]. Detailed study of MSC biology and proliferation, as well as variations due to culture conditions, may further extend the number and potential efficacy of MSCs.

Skeletogenic differentiation of mesenchymal stem cells

MSC differentiation can be controlled by a number of factors, including growth factors, hormones, and extracellular matrix (ECM) molecules, working through various signaling pathways and mechanisms. For instance, of relevance to cartilage tissue engineering, in vitro chondrogenesis of MSCs, or cartilage formation, is accomplished by culturing the cells in a three-dimensional (3D) culture condition, either as pellet culture, high-density micromass cultures, or within biomaterial scaffolds in the presence of a member of the transforming growth factor-β family (TGF-β1, TGF-β2, TGF-β3, or BMPs), insulin growth factor (IGF), Indian hedgehog (Ihh), parathyroid hormone–related protein (PTHrP), FGF, and Wnt/β-catenin [113,119,120]. Supplementation with dexamethasone also promotes the expression of chondrocyte phenotype [121]. Other nontraditional chondrogenic induction approaches, such as biomechancial conditioning, electrostimulation, and oxygen tension, have also been shown as alternatives to chemical agents to promote MSC chondrogenesis [122–124]. Chondrogenesis is demonstrated histologically as well as on the basis of the expression of genes encoding cartilage matrix components, including aggrecan, collagen types II and IX, and cartilage oligomeric matrix protein (COMP) [125]. Control of MSC chondrogenesis will be discussed in more detail in the "Cartilage tissue engineering" section.

Osteogenesis, that is, bone formation, can be induced in vitro by treating MSCs with the synthetic glucocorticoid dexamethasone, β-glycerophosphate, ascorbic acid, and 1,25-dihydroxyvitamin D_3. Alkaline phosphatase (ALP) activity and calcification of the ECM are typical markers used for detecting osteogenesis and mineralization, respectively. As in chondrogenesis induction, dexamethasone is similarly introduced during osteogenic induction of MSCs, and it has been reported to stimulate MSC proliferation and increase ALP activity [126]. Matrix mineralization only occurs in the presence of β-glycerophosphate, ascorbate, and dexamethasone and is further enhanced by 1,25-dihydroxyvitamin D_3 [127]. Although used less uniformly, a number of growth factors, including IGF-1, epidermal growth factors, and vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), have also been reported to contribute to MSC osteoinduction to varying extents [128]. In addition to treatment with growth factors, osteogenesis can be stimulated in vitro by other biophysical and biochemical stimuli, including mechanical stimulation, pulsed electromagnetic field [129], hydrostatic pressure, oxidative stress, or immunomodulators [130–136]. Such models, in addition to offering insight into bone-forming processes, can also be used to analyze various injury mechanisms and to develop potential therapies.

Plasticity of mesenchymal stem cells

Plasticity/transdifferentiation refers to the ability of a cell type committed to and progressing along a specific developmental lineage to switch into another cell type of a different lineage through epigenetic reprogramming. As demonstrated by several studies, terminally differentiated cells can switch their phenotype under appropriate stimulations. For example, chondrocytes can become osteocytes [137,138], and adipocytes can convert to osteoblasts [139–141]. Since all of these cells are the mature progenies of MSCs, the conversion between each other might reflect the plastic property of MSCs. By using an in vitro differentiation strategy, we have demonstrated that human MSCs precommitted to a given mesenchyme cell lineage can transdifferentiate into other cell types in response to inductive extracellular cues [22]. Understanding the

molecular mechanisms that control the transdifferentiation potential of MSCs will facilitate the identification of regulatory factors, thus providing tools to manipulate adult stem cells for cell-based tissue engineering applications. Recent evidence suggest that mesenchymal cell populations contain a subpopulation of multilineage-differentiating stress enduring (MUSE) cells that possess pluripotency and may account for some of the plasticity observed [142]. Moreover, variations in the nanotopographical structure of materials supporting seeding cells can also lead to different cell fates [143].

In addition, plasticity also refers to the multiple differentiation lineages associated with MSC cell fate commitment during tissue morphogenesis that takes place as a function of development and disease pathogenesis in vivo. MSCs participate in the remodeling and repair of many tissues and commit to different cellular lineages dependent on the action of genetic, molecular, and microenvironmental mediators. For example, during aging, MSCs are more inclined to undergo differentiation into adipocytes rather than osteoblasts [144]. Besides the well-characterized TGF-β/BMP/WNT signaling pathways, other factors that regulate the plasticity of MSCs in the bone marrow include noncoding RNAs, such as miR-188 and lncRNA-Bmncr [145,146].

Mesenchymal stem cell heterogeneity

A high degree of variability was observed in MSCs, dependent on donors and tissue sources, including microanatomical locations within a given tissue [147]. Such cellular heterogeneity is reflected as differences in cell morphology, rate of proliferation, onset of senescence, differentiation potential, and functionality [148].

Individual colonies derived from single MSC precursors exhibit a heterogeneous nature in terms of cell proliferation and multilineage differentiation potential. For instance, only a minor proportion of colonies (17%) derived from adult human bone marrow continued to grow beyond 20 population doublings, while the majority of the colonies exhibited early senescence [80]. There is also a marked difference in the differentiation capacity of MSC colonies. For example, Pittenger et al. [5] reported that only one-third of the initial adherent bone marrow–derived MSC clones are multipotent to differentiate along the chondro-, osteo-, and adipogenic pathways (osteo/chondro/adipo). Furthermore, nonimmortalized cell clones examined by Muraglia et al. [78] demonstrated that 30% of the in vitro-derived MSC clones exhibited a tri-lineage (osteo/chondro/adipo) differentiation potential, while the remainder displayed a bilineage (osteo/chondro) or unilineage potential (osteo). These observations are consistent with other in vitro studies using conditionally immortalized clones. Even within a clonal colony, cellular phenotype can be highly heterogeneous [149]. Kuznetsov et al. demonstrated that only 58.8% of the single colony–derived clones had the ability to form bone within hydroxyapatite (HAP)–tricalcium phosphate (TCP) ceramic scaffolds after implantation in immunodeficient mice [150]. Similar results were reported by using purer populations of MSCs maintained in vitro [80] as well as human trabecular bone–derived MSCs [31]. Taken together, these results suggest that MSCs are heterogeneous with respect to their developmental potential and place a significant challenge on selecting the most potent cells for clinical application in tissue regeneration.

There exist a number of inconsistencies and discrepancies among currently published results on MSCs, perhaps reflecting the heterogeneity of the cell population, different isolation, and culture methods, as well as the different stimuli used for the differentiation procedures [20]. There has not been a single MSC-specific marker to unequivocally identify MSCs, and there is heterogeneity in MSC populations. The emerging tools of single-cell isolation and analysis [151] should yield better profile information about cellular heterogeneity and characteristics of different subpopulations, to improve the selection of the proper MSC candidates in clinical applications [152].

Mesenchymal stem cell effect on host immunobiology

Ideally, autologous MSCs would have advantages over allogeneic MSCs for regenerative medicine as autologous MSCs pose few immunological complications. Due to the low abundance of MSCs and the possible decrease in quantity as well as quality of the MSCs with age and disease, the use of allogeneic MSCs for replacing or repairing damaged tissues needs to be taken into consideration. Before this is deemed acceptable or feasible, it is important to understand how allogeneic cells elicit host immunological reactions. Allogeneic cells are normally detected and deleted by the host immune systems. However, MSCs have been surprisingly different in this aspect and offer several advantages, including possible immunomodulatory, including immunosuppressive, effects on the host response.

The first immunological advantage of MSCs is that they are hypoimmunogenic and can evade host immune system, as shown by several in vitro experiments [153]. This makes MSCs attractive for allogeneic transplantation whose major limitation is host immune rejection. MSCs express low (fetal) to intermediate (adult) major histocompatibility complex (MHC) class I molecules and do not express MHC class II molecules on their cell surface [154,155]. The lower expression of MHC class I molecules helps to protect MSCs from deletion by natural

killer (NK) cells. The lack of surface MHC class II expression gives the MSCs the potential to escape recognition by alloreactive CD4 + T cells. MSCs contain an intracellular pool of MHC class II molecules that can be mobilized onto cell surface when treated with interferon-γ (IFN-γ). However, induced surface expression of MHC class II still does not render the MSCs immunogenic [155]. After differentiation, MSCs continue to express MHC class I but not class II molecules on their cell surface and continue to be nonimmunogenic [154,155]. Nevertheless, it has been shown that although allogeneic MSCs are less immunogenic than other cell types, they do not completely evade immunological recognition [156]. Indeed, the survival period of allogeneic MSCs is significantly shorter than autologous MSCs [157].

MSC-mediated immune modulation is induced via both paracrine and direct cellular interactions with monocytes/macrophages, dendritic cells, T cells, B cells, and NK cells. Experimental evidence suggests that MSCs can interact directly with immune cells and modulate and suppress alloreactivity. MSCs inhibit T cell proliferation in vitro [158–160], do not seem to express costimulatory molecules, CD40, CD40 ligand, CD80, and CD86 (formerly B7–1 and B7–2, respectively), and probably do not activate alloreactive T cells [160]. In fact, MSCs suppress T cell activation and proliferation. For example, MSCs have been shown to suppress CD4 + and CD8 + T cells in mixed lymphocyte cultures [159,160]. Even though T cell proliferation can be induced by exogenous costimulation, when they are cocultured with MSCs in the presence of a stimulant, T cell proliferation is not observed [160]. MSCs can also induce apoptosis of activated T cells but not resting T cells [161]. MSCs also facilitate the formation of regulatory T cells (Tregs) in vitro and in vivo. Tregs are involved in immune homeostasis and are implicated to be a major effector cell type responsible for MSC-mediated immune modulation [162,163]. Supporting these observations, Bartholomew [158] showed that allogeneic baboon MSCs inhibited the proliferation of lymphocytes in vitro and prolonged skin graft survival in vivo. In addition to their effects on T cells, MSCs can also affect dendritic cell differentiation and maturation and interfere with their function [164,165]. MSCs have also been shown to inhibit polarization of macrophages and monocytes toward a proinflammatory phenotype (M1) and facilitate their polarization toward an immunomodulatory (M2) phenotype [166,167]. They also alter the phenotype of NK cells and can suppress the proliferation, cytokine secretion, and cytotoxicity of these cells against MHC class I targets [168]. By directly interacting with B cells, MSCs are also capable of reducing plasmablast formation and induce a regulatory phenotype in B cells [169].

In addition to cell–cell interaction mediated inhibition, MSCs are capable of secreting soluble factors to create a local immunosuppressive environment. These factors have been shown to include hepatocyte growth factor (HGF), TGF-β1, IL-10, and prostaglandin E2 [159,162]. When anti-HGF and anti-TGF-β1 antibodies are included, MSC inhibition on T cell proliferation is lifted [159]. IL-10 is a cytokine for Tregs and can suppress inflammatory immune response. Similarly, MSCs are shown to produce IL-10 either constitutively, or in coculture, and MSCs can mediate the suppressive activities partially through secretion of IL-10 [162,164,170]. Taken together, these in vitro findings suggest that MSCs can interact with the various subsets of cells of the immune system, alter the response of the immune cells, and shift the response from a proinflammatory response to an antiinflammatory response, possibly through the inhibition of the proinflammatory cytokines, such as IFN-γ and tumor necrosis factor-α (TNF-α), and stimulation of the immunosuppressive cytokines, including IL-10 and prostaglandin E2 [162]. Other identified crucial antiinflammatory factors secreted by MSCs include TNF stimulated gene 6 (TSG6) and indoleamine 2,3-dioxygenase (IDO). The latter was implicated to be partially responsible for NK cell suppression [162] and T cell activation [171]. MSC-derived TSG6 was demonstrated to attenuate inflammatory response and promote tissue repair in acute lung injury [172], peritonitis [173], and arthritis [174]. Another emerging field of research concerns extracellular vesicles (including exosomes), which carry microRNA, mRNAs, and other proteins and have been shown to be at least partially responsible for the immunomodulatory properties and therapeutic effects of MSCs [175–179].

The role of secreted factors in the immunoregulatory action of MSCs remains an actively investigated area [180,181]. Contradictory results exist as to which factors are important for this function. For example, although a role for IL-10, TGF-β, and prostaglandin E2 has been suggested, in other studies, none of these factors was found to be responsible for the immunosuppressive action of MSCs [160].

Recent studies have further reported that apoptotic, heat inactivated (dead), or even fragmented MSCs have immunomodulatory properties [182]. These were implicated to be mediated via monocytes and macrophages, which phagocytose dead MSCs and their fragments, leading to their polarization toward an antiinflammatory M2 phenotype [183]. A potential mechanism implicates that IDO is released within phagocytes, which in turn leads to the inhibition of T cells [184–186]. Irrespective of the exact mechanism of action, these findings suggest the feasibility of transplantation of MSCs between MHC incompatible individuals.

The in vitro studies showing that MSCs possess immunomodulatory and immunosuppressive activities suggest that MSCs can be potentially used in vivo for enhancing the engraftment of other cells and tissues (e.g., hematopoietic stem cells) or for the prophylactic prevention and even possibly as a treatment of graft-versus-host-disease, to prevent rejection, and promote transplant and patient survival. Other potential applications include inflammatory bowel disease, multiple sclerosis, liver and kidney injury, just to name a few. Indeed, a search of clinicatrials.gov (August 19, 2019) returned 194 studies using the terms "mesenchymal stem cell" and "immune" [187]. However, before MSC treatment can be used as standard therapy on humans, more in vivo animal studies need to be performed, and the biology and mechanism of MSC immunomodulation effect need to be better elucidated.

Safety of using mesenchymal stem cells for transplantation

MSC transplantation for therapeutic purpose has been under intensive investigation, and many studies have advanced from bench to bedside [188]. There is an abundance of clinical trials and studies using autologous or allogeneic MSCs for tissue regeneration and disease conditions. Most of the autologously derived MSCs, progenitors, and derivatives [189] have been proven to be safe [190], and many applications also show some favorable clinical results, including osteochondral repair [13], rotator cuff repair [191], and knee osteoarthritis [192,193]. For allogeneic cells the survival rate of the transplanted cells decreases dramatically after a short period of time, usually 1−2 weeks, indicating a treatment time window, and the need for multiple administrations during treatment. The allogeneic MSCs have proven to be safe in recurrent uterine adhesion [194], multiple sclerosis [195], and other application scenarios. Currently, the major safety concerns of using MSCs focus on how to reduce the residual animal-derived products present during cell expansion cultures, suppress potential immune reaction and related side effects, and increase cell survival rate after transplantation.

Indeed, the ability of MSCs to undergo extensive self-renewal via proliferation raises some concern as to whether MSCs, after prolonged in vitro culture, can become tumorigenic. For now, there is no strong evidence of the tumorigenic transformation of MSCs; however, it should be noted that endogenous MSCs are usually recruited by tumor cells during tumor growth and progression [196−199]. The immunosuppressive properties, especially the potential systemic immunosuppressive ability of MSCs, bring caution to the use of MSCs under certain clinical conditions, such as cancer. There are distinct effects of MSCs in the initial and progressive stage of tumorigenesis [198], and there are ongoing debates on the use of MSCs for cancer therapy. It has been shown that cotransplantation of an MSC cell line (C3H10T1/2) favors tumor growth of subcutaneously injected B16 melanoma cells in a murine melanoma tumor model [200]. However, this tumor-promoting effect was not observed in another study of coculture of a rat MSC line, MPC1cE, with rat colon carcinoma cells in a gelatin matrix. In the latter case, inhibitory effect of MSCs on the outgrowth of the tumor cells was observed [201]. The effect of MSCs on tumor growth thus requires further investigation in order to minimize or eliminate the potential side effect of therapeutic use of MSCs [202].

Mesenchymal stem cells in musculoskeletal tissue engineering

The musculoskeletal system of the human body is designed to sustain and maintain its form and function in the face of strenuous load bearing demands throughout a normally active life. Form and mechanical strength are vital for the function of this system. The mechanical and biochemical properties of the musculoskeletal system that define its structure and function also define the functional requirements of a tissue-engineered substitute. Successful tissue engineering−based replacement of native tissues will likely require constructs that possess functional properties similar to those of the native tissues to minimize premature failure. There are two basic approaches in tissue engineering. The first one is ex vivo tissue engineering, in which the construct is cultivated in vitro to achieve appropriate functionality before implantation. The second approach is in vivo tissue engineering, in which the construct is allowed to mature in vivo for tissue repair and regeneration. For both approaches, three components govern the eventual outcome of tissue-engineered constructs: *appropriate scaffold, instructive environment, and responsive cells*.

Cartilage tissue engineering

The need for engineered cartilage arises from the fact that while the tissue often functions well through a lifetime of use, over 30% of adults in the United States over 30 years of age have radiographic evidence of cartilage degeneration, with 9%−10% of the US population aged 30 and over suffering symptoms of osteoarthritis of the hip or knee [203,204]. The total cost of osteoarthritis is estimated at $36 billion dollars per year in the United States alone [205], with >200,000 knee replacements performed each year. The intrinsic healing capacity of the native tissue is limited, and given the increasing incidence of osteoarthritis and increasing life expectancy of the population,

there is a growing demand for novel repair strategies. Effective treatment of cartilage injuries may eliminate or forestall the need for joint replacement, thus enhancing the quality of life.

General properties of articular cartilage

Articular cartilage is the dense white tissue that lines the surfaces of joints and functions to transmit the high stresses associated with joint motion. The tissue consists of both a solid ECM component as well as a fluid phase [206,207]. The solid ECM is composed of a dense network of specialized molecules that engender the unique mechanical properties of the tissue. Collagen content (predominantly collagen type II) of the tissue ranges from 5% to 30% by wet weight while proteoglycan content ranges from 2% to 10%. In addition to these major elements the ECM also includes numerous minor collagens (e.g., types VI, IX, and XI), and linking molecules (COMP, hyaluronan, link protein, and fibronectin). The highly charged ECM and the proteoglycans, in particular, trap a large amount of water within the cartilage matrix, which comprises the fluid component of cartilage matrix. Together, these elements make up the fluid-filled fibrous network with larger collagen fibers interwoven throughout an array of large proteoglycan aggregates (aggrecan core protein with its covalently linked keratin and chondroitin sulfate moieties) attached to long hyaluronan chains.

The exact composition of cartilage changes slightly but sequentially as one progresses from the surface zone of the articular cartilage to the calcified zone that interacts with the subchondral bone. The surface zone, which is well adapted to tensile forces, comprises relatively little matrix with finer fibrillar structure rich in the proteoglycan lubricin arranged parallel to the articular surface as revealed by polarized microscopy and the squamal morphology of the cells in this region. The deep zone, well adapted to compressive forces, comprises larger collagen fibrils with greater cross-linking arranged perpendicular to the articular surface. These core constituents change with age, resulting in the emergence of adult functional properties, and deteriorate with disease processes, such as osteoarthritis [208–210].

The cellular component of cartilage, the chondrocytes, comprises less than 10% of the tissue volume [211]. The sparse distribution of chondrocytes belies their critical importance in maintaining the balance between anabolic synthesis of ECM constituents and continual remodeling and degradation over time [212]. Chondrocytes display a rich transcriptional profile in situ [213], reflective of their specialized cartilaginous phenotype, which is lost when these cells dedifferentiate with expansion in monolayer culture [214]. This process may be substantially reversed, with chondrocytes redifferentiating toward their mature phenotype when returned to 3D culture [215]. However, injury and diseased conditions may lead to the cells undergoing "irreversible" phenotypic changes such as the expression of collagen type I that leads to fibrosis, and the expression of matrix metalloproteinases (MMPs) [216], thus reducing the mechanical properties of the matrix comprising repair tissue or engineered cartilage, and harkening matrix and tissue degeneration.

The mechanical properties of articular cartilage are complex and underlie its ability to act as a low friction, weight-bearing surface over a lifetime of use. These properties are a result of both the solid (matrix) and fluid phases of the tissue. Regarding the solid, matrix phase, the dense, negatively charged, proteoglycan-rich ECM results in an equilibrium compression modulus of 0.2–1.4 MPa [217,218]. Meanwhile, the collagen content engenders a tensile modulus that is higher, ranging from 1 to 30 MPa [219,220]. Other structural elements of cartilage, including collagen type IX [221] and COMP [222], participate in the cross-linking of the collagen type II ECM, increasing matrix connectivity.

The highly charged ECM and the proteoglycans, in particular, trap a large amount of water within the cartilage matrix [223]. These electrostatic forces and the physiological cyclic loading (0.1–2 Hz) of the tissue maintain elevated interstitial fluid pressure. Fluid pressurization increases with contact and supports >90% of applied stress, shielding the matrix from excess deformation [224]. This fluid pressurization also results in a higher dynamic modulus than equilibrium modulus, stiffening the tissue with higher frequency and/or higher rate of loading. Finally, this fluid pressurization, coupled with molecules that participate in boundary lubrication, maintains the frictional coefficient of cartilage at extremely low values (lower than ice on ice), further protecting the tissue from excess wear with physiologic use [225,226].

Cells for cartilage tissue engineering

In the quest to repair or replace damaged articular cartilage, any replacement material, tissue, or their composite must be fabricated to retain the complex mechanical properties described previously. The development of such a material begins with the consideration and combination of appropriate and responsive cells, biocompatible and mechanically conductive scaffolds, and inductive environment for the optimal differentiation and proliferation of the cell type of interest. As discussed in this and other chapters, MSCs derived from various adult tissues have emerged as promising cell sources because of the ease with which they can be isolated and expanded and their multilineage differentiation capabilities.

When tissue engineering was originally proposed as a strategy for repairing diseased or damaged tissues [227],

it was believed that articular cartilage would be one of the first successes in this new field, owing to its relatively simple composition (possessing a single cell type) and lack of neural and vascular supply. First approaches focused on the chondrocyte itself, postulating that as these cells make and maintain matrix in vivo, exogenous cells should be able to reconstitute the tissue when implanted [228]. The first-in-patient successes demonstrated enhanced repair when high density chondrocyte solutions were transplanted to focal defects beneath a periosteal flap [229], a procedure now commonly referred to as autologous chondrocyte implantation (ACI) [230]. It stands as the first clinically available tissue-engineering strategy to enhance cartilage repair, and later was commercialized as the Carticel method by Genzyme Biosurgery and other brands and companies [231]. While questions remain regarding the cost-effectiveness of this approach, as compared to the simple bone marrow stimulation approach [232], the treatable defect size and quality of repaired tissue [233], and the over 20 years of good and satisfied follow-up results [234] make it a reliable and indispensable method for cartilage repair [231]. With subsequent technology development, scaffold or matrix has been incorporated into the original cell-based approach for better cell retention and as a substitute for the periosteal flap (referred to as matrix-assisted ACI or MACI) [235].

Although significant progress has been made in the production of engineered cartilage constructs with chondrocytes, several significant impediments exist that limit their clinical application. For instance, autologous chondrocytes are present in limited supply and are often of an aged and/or diseased state in patients presenting with osteoarthritis [236,237]. One opportunity for overcoming such concerns is the use of chondroprogenitor cells, such as tissue-specific progenitors [13] or progenitors derived from adult MSCs [231]. As described previously, these cells may be readily obtained from the adult bone marrow and other tissues and retain self-renewal ability and multipotency. These cells are expandable in culture [82] and can be grown in sufficient numbers to populate tissue-engineered cartilage. Recently, Jiang et al. reported the successful application of human autologous cartilage–derived stem/progenitors to treat large-sized osteochondral defects (6–13 cm^2) in patients [13].

The use of MSCs for cartilage regeneration and tissue engineering has shown enormous potential and initial success. MSCs have been used as cellular therapeutics directly, via injection into the joint space (with and without a carrier matrix) in an undifferentiated state [238–240] and after differentiation down a cartilage differentiation factors that drive their own chondrogenesis after implantation [241]. Of note, one current surgical strategy for enhancing the repair of cartilage defects is via microfracturing of the subchondral bone. This technique, also referred to as bone marrow stimulation [231], provides entrance for marrow elements, including MSCs, to the wound site and has been shown to generate an enhanced, albeit fibrous, repair response [242].

Mesenchymal stem cell chondrogenesis

Mesenchymal stem cell chondrogenic potential

Control of chondrogenesis in MSCs is a complex and developing research area, with much of our understanding of the relevant molecules and processes stemming from a continuing elucidation of the events that control healthy cartilage homeostasis as well as cartilage formation in the developing limb. Elements including soluble factors such as growth factors, cytokines, hormones; various intracellular signaling pathways and transcription factors; environmental factors such as mechanical loading and oxygen levels; and seeding density all affect chondrogenic differentiation of MSCs.

The standard in vitro system of MSC chondrogenesis involves a 3D culture of MSCs under the stimulation of a suitable chondrogenic stimulus. High density cultures of pellet or micromass are frequently employed, modeling early chondrogenesis in development where condensation of the early progenitor cells initiates the cascade of events leading to cartilage formation [243]. These MSCs can then effectively differentiate under standard chondrogenic conditions (serum-free medium supplemented with insulin–transferrin–selenium, ascorbate, proline, dexamethasone, and TGF-βs). In addition, MSCs may also be seeded on various 3D scaffolds to induce chondrogenesis. While the maintenance of a spherical shape in these scaffolds may be enough to shape a chondrogenic phenotype by MSCs, a chondroinductive growth medium is still required to realize the terminal differentiation and cartilage matrix accumulation of these cells. The hallmarks of an articular chondrocyte are initially high levels of Sox9 expression followed by ultimately high levels of collagen types II, IX, and XI and concomitant expression of aggrecan, COMP, and link protein, among others. Together these extracellular components produce a cross-linked and highly hydrated matrix surrounding the cells and their pericellular and intercellular matrix. This phenotype represents the prehypertrophic state of cartilage in the parlance of developmental biology describing the growth plate, the cartilage anlage of the endochondral skeleton [243].

MSCs that have undergone chondrogenic differentiation assume a chondrocyte-like phenotype characterized by increases in proteoglycan deposition and expression of aggrecan, COMP, and collagen type II [244] as described previously, and microarray analysis has shown increased gene expression of a large number of other cartilage ECM

elements as well [22,245,246]. A major challenge to articular cartilage engineering is to prevent the apparently natural progression of the cells down the path of chondrocyte hypertrophy, matrix mineralization, and apoptosis that promotes the ossification of the tissue to mature bone, as observed during the endochondral ossification process of embryonic skeletogenesis [243]. In this latter process, cells express Runt-related transcription factor 2 (Runx2), collagen types I and X, and matrix degradative enzymes such as MMPs (e.g., MMP13). Interestingly, these markers are also characteristic of chondrocytes in osteoarthritic cartilage [247] and, if expressed in engineered cartilage, are presumed to result in matrix degradation, and scaffold and construct failure.

Signaling in mesenchymal stem cell chondrogenesis

Growth factors that have regulatory effects on MSCs include members of the TGF-β superfamily, the IGFs, the FGFs, and the PDGFs. Among these growth factors, members of the TGF-β family, including TGF-β1, TGF-β2, and TGF-β3, as well as BMPs are the most potent inducers to promote chondrogenesis of MSCs. For human bone marrow–derived MSCs, TGF-β2 and TGF-β3 were shown to be more active than TGF-β1 in promoting chondrogenesis [244]. BMPs, known for their involvement in cartilage (as well as in bone) formation, act alone or in concert with other growth factors to induce or enhance MSC chondrogenic differentiation. For example, BMP2, BMP4, or BMP6, combined with TGF-β3, induced the chondrogenic phenotype in cultured human bone marrow–derived MSC pellets, with BMP2 seemingly the most effective [248]. Other growth factors, such as IGF, FGF, and PDGF, are important signaling molecules that mediate chondrocyte physiology rather than promoting chondrogenesis of MSCs and therefore commonly work with TGF-βs to promote chondrogenesis and enhance chondrocytic activities of differentiated MSCs. The promitotic activity of the FGFs has also been exploited for cell expansion purposes [249]. Interestingly, FGF-2-supplemented human MSCs proliferated more rapidly and exhibited greater chondrogenic potential than untreated controls [250]. Canonical Wnt signaling has been shown to enhance MSC differentiation [251], and Wnt signaling in chondrogenesis has been shown to crosstalk with TGFβ signaling [252–254]. The current challenge in finding the most efficient growth factor(s) for MSC chondrogenesis is that the regulatory effects of signaling molecules are dependent on property, dose, and timing of the molecules administered to the cells. This may explain some of the contradictory results regarding the effects of specific growth factors on chondrogenesis. Furthermore, the exact mechanisms of articular cartilage development in vivo remain incompletely understood; however, we may surmise that it occurs in a very complex biochemical environment, and that some level of this complexity must be replicated in vitro in order to produce functional articular cartilage. Replication of this complexity begins in part with the application of growth factor cocktails, applied combinatorially or sequentially, and as the effects of growth factors are changed in the presence of other growth factors and environmental cues, the exact repertoire of prochondrogenic formulations is likely to remain unsettled before the field achieves clarification.

Growth factors act on cells and induce various intracellular signaling pathways to coordinate transcription factors and change cellular phenotype. The most important molecule intrinsic to the assumption of the cartilaginous phenotype is the transcription factor, Sox9. The role of Sox9 in cartilage formation was first observed in the process of cellular condensation in the developing mouse limb, and its presence is thought to be required for cartilage formation [255]. The expression of Sox9 is considered a master regulatory step of chondrogenesis, defining the commitment of a mesenchymal cell, such as the MSC, down the chondrogenic lineage. The action of Sox9 is enhanced by the related molecules, Sox5 and Sox6 [256], with recent evidence suggesting that the exogenous introduction of the combination of the three is sufficient to induce chondrogenesis in a variety of cell types [257]. In human bone marrow–derived MSCs, exogenous expression of Sox9 led to increased proteoglycan deposition and expression of link protein [258,259]. It has been shown that the addition of BMP2 resulted in dose-dependent increase in Sox9 expression in C3H10T1/2 cells, a mesenchymal progenitor cell line [260].

In terms of clinical applications, BMP2 and BMP7 (also known as OP-1) have been approved by the US Food and Drug Administration (FDA) for clinical use [261], thus rendering them good candidates for preclinical studies of cartilage tissue engineering. Recent studies have shown that the nature of the effective chondrogenic growth factors and their combinations are slightly different for MSCs-derived from different tissue sources, for example, bone marrow, adipose, induced pluripotent stem cell (iPSC)-derived progenitors, and tissue-specific progenitors. For example, bone marrow–derived MSCs are primarily responsive to TGF-β1 and TGF-β3 [244,262,263], whereas adipose-derived MSCs require BMP6 in addition to TGF-βs to achieve adequate chondrogenesis [264,265]. Adding BMP4 to TGF-β supplemented chondro-inductive medium can boost chondrogenic differentiation of bone marrow or cartilage-derived cells [13,266]. These differential requirements likely reflect the inherent differences in tissue origin [152], and proper combination and strategy of applying growth factors thus depends on the source of MSCs and the delivery method used for cartilage tissue engineering.

In addition to growth factors a number of "physical" environmental factors also influence MSC chondrogenesis. These include oxygen tension, mechanical loading, and hydrostatic pressure. Articular cartilage is an avascular tissue, therefore nutrients and metabolites are provided via diffusion from the synovial fluid and the subchondral bone, aided by the movement and resulting fluid flow in the joint. Oxygen tension in the deep zones of articular cartilage has been reported to be between 1% and 2% with an increasing oxygen gradient toward the articular surface [267,268]. Hypoxia is a positive regulator of the chondrocytic phenotype and MSC chondrogenic differentiation [269–271]. Regular, cyclic loading of cartilage is vital to its homeostasis in vivo, and cyclic loading has been shown to be chondroinductive in vitro [272–274] and even prevent the induction of hypertrophic markers in vitro [275]. Hydrostatic pressure itself, a component of the increased interstitial pressure experienced by cartilage under load, is chondro-stimulatory [276], although not in isolation, and its effects are quite variable between individuals [277]. One interesting way to regulate chondrogenesis of MSC is to use biophysical signals, such as short-term high voltage electric signals. Application of nanosecond pulsed electric fields to precondition MSCs can enhance the differentiation ability in subsequent in vitro cultures [278,279]. These examples illustrate the importance of understanding normal cartilage homeostasis in order to engineer better articular cartilage.

Scaffolds for cartilage tissue engineering

The 3D substrate upon which the cells are grown can have profound effects upon MSC differentiation. Considerations include the material composition, its structure and topography, and its biodegradability. In general, scaffolds may be made of natural or synthetic biomaterials and may be in the form of hydrogels, sponges, fibrous meshes, and nanofibers. Only some of the many available scaffolds are appropriate for cartilage tissue engineering. Among artificial materials, poly-glycolic acid (PGA), poly-L-lactic acid (PLA), poly-ε-caprolactone (PCL) polyethylene oxide, and polyethylene glycol (PEG), among others, have shown potential in cartilage tissue engineering [280–286]. These polymers may be employed as either sponges formed through phase separation and salt leaching or as micro- or nanofibers formed by electrospinning. The small diameters of nanofibers closely match the geometry of collagen fibrils comprising a natural cartilage matrix, making these structures a potentially useful biomimetic scaffold (Fig. 49.3) [287,288]. The high porosity, high surface-to-volume ratio and their unique mechanical properties account for the popularity of these structures in cartilage engineering as well. In recent studies, natural materials such as chitin [289], collagens [290], hyaluronan [291], silk [292,293] among others, have been processed into nanofibrous scaffolds. These biomaterials have the added value of providing natural epitopes for cell attachment as well as sites for covalent attachment of osteogenic molecules. Cospinning different materials together can be useful in creating complex surface topographies, greater porosity, increasing biological activities, and adjusting mechanical properties of the resulting scaffold [294]. Finally, fibers may be spun directionally to yield the proper matrix alignment as well as potentially transmit or absorb mechanical forces necessary to the protection and function of the tissue.

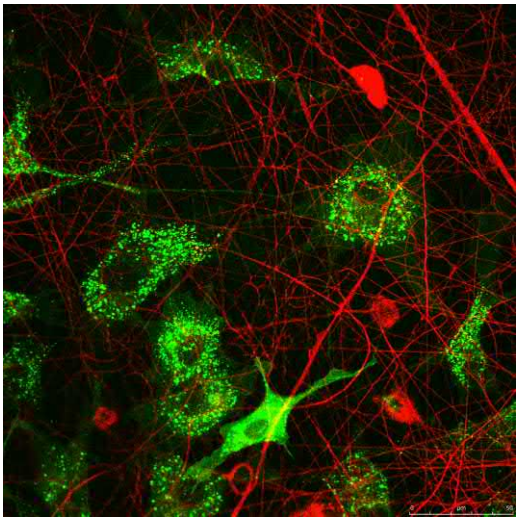

FIGURE 49.3 Confocal laser scanning fluorescence microscopy of adult human MSCs (green) seeded on to an electrospun nanofibrous scaffold (red). MSCs appear to be well integrated within the nanofibrous matrix, interacting with individual fibers via cellular processes, as well as filling the "pores" within the scaffold. Scale bar = 50 μm. *MSCs*, Mesenchymal stem cells.

In addition to fibrous scaffolds, various biocompatible hydrogels have also been applied for the in vitro and in vivo growth of cartilage constructs. Hydrogels are 3D, hydrophilic, polymeric networks capable of retaining large amounts of water or biological fluids and commonly used to mimic the chondrogenic environment. Frequently used hydrogels include synthetic materials such as PEG, self-assembling peptides, and natural materials, including agarose [207,295,296], alginates [297–299], the cartilage matrix components collagen type I [300–302], collagen type II [303], hyaluronan-based gels [304,305], and fibrin gels [306,307]. The exploration of each of these scaffolds has contributed to our understanding of MSC chondrogenesis for tissue engineering.

Agarose gels have been employed extensively in cartilage tissue engineering and are unique in that they offer cells no epitopes for adhesion. The agarose maintains the cell in a spherical shape, which alone has been shown to maintain and even promote chondrogenesis.

The otherwise inert agarose has been useful in elucidating the effects of mechanical loading, TGF-β exposure, and differences between chondrocytes and MSCs.

Synthetic PEG hydrogels, although relatively inert, have been shown to support cartilage tissue formation by both chondrocytes and MSCs [308,309]. Unlike many other synthetic polymers, PEG may be modified to include arginine−glycine−aspartic acid (RGD) and other bioactive peptides [310,311] to enhance viability [312] and chondrogenic differentiation of encapsulated cells [313], and to control the mechanical [314] and degradation [315] properties of the hydrogel itself. These features have made it a staple of tissue engineering. The inclusion of cartilage matrix molecules or peptide derivatives greatly enhances the PEG scaffold support of chondrogenesis and may be tuned to create multilayered constructs [316−318]. The addition of hyaluronan is particularly potent in inducing MSC chondrogenesis [319]. A natural component of cartilage, hyaluronan is also functionally involved in biological processes, including cell proliferation, morphogenesis, inflammation, and wound healing. Hyaluronan hydrogels also support chondrogenic differentiation of MSCs, making it a promising scaffold for cartilage regeneration [320].

Recent work in our laboratory has led to the development of photocrosslinkable, gelatin-based hydrogel materials that incorporate biocompatibility, mechanical stiffness, high cell viability, and adaptability to clinical imaging guided 3D bioprinting, with the ability of live cell encapsulation during scaffold fabrication [123,321−323]. These materials have allowed efficient MSC-based cartilage tissue engineering and have been applied for the repair of cartilage defect in an in vitro model [324], as well as the construction of an osteochondral chip that functions as an in vitro microphysiological system to simulate the osteochondral junction of an articular joint [325,326].

Factors influencing outcomes of tissue-engineered cartilage

Since the quality of the ECM and the mechanical properties of engineered constructs generally fall short of the characteristics of native cartilage, many studies have focused upon the combination of chondrogenic stimuli. The evolution of a tissue-engineered construct is a time-dependent process, controlled by numerous (and sometimes unanticipated) factors. These factors include an evolving cell phenotype, changing biochemical and biophysical environments, and continuous remodeling of the matrix from the engineered scaffold to native ECM. Two philosophies govern the engineering of each component. On the one hand, tissue engineers may strive to build and control each component throughout the development of the construct. On the other hand, components and processes may be permitted to proceed as naturally as possible in situ. In both cases a perfect scenario involves the development of all construct components in unison to ultimately form a functional tissue. The fact that no tissue-engineered construct based upon MSCs has successfully repaired articular surfaces over the long term shows that such a coordinated development has not been achieved. Thus it is imperative that we seek to understand each process better, particularly as they relate to each other.

In recent years, there has been rapid growth in the number of studies analyzing the synergistic effects of various chondrogenic stimuli, covering

1. different growth factor combinations;
2. scaffolds comprising complex materials with different mechanical, biological, and biodegradation properties;
3. the inclusion of mechanical stimulation and/or hypoxia to the differentiation equation; and
4. the inclusion of various biological matrices, including devitalized tissue matrices and blood fractions including platelet-rich plasma.

While clearly all components of the tissue-engineered construct are under continuous study and improvement, the development of biomaterial scaffold in particular is undergoing rapid evolution. Investigators have focused on how to recapitulate the multiple roles of native ECM, including its roles in structural integrity, mechanotransduction, and repository and coreceptor for various signaling molecules. Toward this end, scaffolds have been decorated with peptide sequences derived from the bioactive motifs of collagen, fibronectin, and laminin (among others) through adsorption or covalent bonding. These peptides have also been modified to include substrate sequences for specific proteolytic enzymes to allow targeted release, a property which is particularly useful when nondegradable scaffolds, such as agarose, are used.

Most bioresorbable scaffolds undergo a degree of degradation that is essentially incomplete during tissue regeneration. Growth factors may be incorporated in the scaffolds using microparticles of select composition, micelles, and composite structures with specific time-release profiles to deliver changing combinations of stimulatory factors to enhance the chondrogenic phenotype of the MSCs. A major advance in the use of hydrogels has been the development of photo-polymerizable monomers that make hydrogels amenable to photo-stereolithography. This technique allows the design and fabrication of detailed internal and surface microarchitecture of large scaffolds such as those required for joint resurfacing. With precise spatial control, scaffolds can be loaded with micro- or nanoparticles of varying composition, tuned to release encapsulated growth factors at specified times. Our recent work showed that the incorporation of

graphene oxide in the photocrosslinked gelatin hydrogen promotes chondro-inductive factor-free chondrogenic differentiation of MSCs, presumably due to the highly efficient adsorption of endogenously produced growth factors by the MSCs, resulting from the high surface area and adsorptive characteristics of the graphene oxide nanoparticles [327]. Thus spatiotemporally specific differentiation of the seeded MSCs can be controlled to approximate and recreate the zonal organization of articular cartilage, osteochondral plug, or musculoskeletal entheses.

Despite such excellent design and fabrication strategies, many challenges remain in using such large complex scaffolds, including enhancement of cell survival as well as efficient and spatially targeted cell seeding. The latter may be largely overcome by centrifugation or vacuum aspiration, traditionally used to seed fibrous scaffolds, or direct incorporation of cells within the scaffold during fabrication, a technique commonly used with hydrogels. In the bottom-up approach to tissue engineering using photo-stereolithography, another challenge is the cytotoxic conditions generated by the UV-activation of photoinitiators during the polymerization of the monomer solution. A solution has recently been reported by the development of a visible light-activatable photoinitator-based method of projection stereolithography [321].

Bone tissue engineering

Bone has a vigorous potential to regenerate itself after damage; however, the efficacious repair of large bone defects resulting from resection, trauma, or nonunion fractures still requires the implantation of bone grafts. Five hundred thousand surgical cases of bone grafting procedures are performed annually [328], and the demand for bone grafts is expected to be even greater over the next decade as the population ages. Natural bone grafts have been used extensively in clinical settings. Autografts are considered the gold standard for bone implantation due to their robust performance in osteoinductivity, osteoconductivity, and osteogenesis, as well as their advantage of immunocompatibility over allografts and xenografts. However, complicating issues, such as donor site morbidity, risk of infection, and the availability of bone tissue of the correct size and shape, limit the use of autografts in orthopedic applications. One possible remedy for the shortage of bone grafts is a functional tissue-engineered bone graft possessing one if not all of the following properties—an osteoconductive matrix, osteoinductive factors, and osteogenic cells [328]. Within this context, MSCs represent an attractive cell type for bone tissue engineering due to their easy accessibility, self-renewal and avid bone forming ability, acceptable genomic stability, and minimal ethical complications for clinical application. In bone tissue engineering, MSCs can either be used together with scaffold carriers or through scaffold-free approaches, such as systemic/local injection or cell sheet-based therapy, in the presence or absence of various osteoinductive factors [329,330].

Traditionally, MSCs are induced to undergo osteogenesis in vitro by the use of chemical supplements as described earlier (see the "Skeletogenic differentiation of mesenchymal stem cells" section) before in vivo application. Growth factors, such as Wnt family members, are noteworthy as they are well-established signals that can regulate multiple bone−related biology processes [114]. Interestingly, different Wnt family members can exert different roles in MSC osteogenesis. For example, in vitro exposure of MSCs to Wnt3a-conditioned medium or overexpression of ectopic Wnt3a inhibits osteogenesis. This was evidenced by dramatically reduced expression of several osteogenic genes, for example, ALP, bone sialoprotein, and osteocalcin, while Cbfa1/Runx2 expression, an early osteo-inductive transcription factor remained unchanged. These results implied that the Wnt3a-mediated canonical signaling pathway could inhibit but was not sufficient in complete blockage of MSC osteogenesis. On the other hand, Wnt5a, a typical noncanonical Wnt member, has been shown to promote MSC osteogenesis. Since Wnt3a promotes MSC proliferation during early osteogenesis, it is very likely that canonical Wnt signaling functions in the initiation of early osteogenic commitment by increasing the osteoprogenitor reservoir, while noncanonical Wnt drives the progression of osteoprogenitor to mature functional osteoblasts. The exact identity and actions of intracellular mediators of Wnt signaling in regulating MSC osteogenesis remain to be elucidated.

In addition to Wnt family members, their pathway modulators, such as Dickkopf-related protein 1 (DKK1) and Sclerostin (SOST), and other growth factors or osteogenesis molecules, for example, TGF-β, BMP, FGF, IGF, PDGF, Ihh, NEL-like protein 1 (NELL-1), slit guidance ligand 3 (SLIT-3) [331,332], Notch, PTH, and PTHrP, have all been shown to regulate MSCs osteogenesis and may be considered for application in bone tissue engineering.

In order for MSCs to generate a successful bone graft, the cells require 3D biomaterial scaffolds to secure them at the implantation site, provide physical protection, and maintain and direct tissue shape. In MSC-based bone tissue engineering, various biomaterial scaffolds have been evaluated for their potential as cell carriers. These scaffolds may be made from natural or synthetic materials that have been fashioned into structures with different shapes and sizes. In general, natural polymers, such as collagens, contain bioactive domains favorable for biological activities involved in tissue regeneration, whereas synthetic polymers, such as poly(α-hydroxyesters),

feature controllable material properties that can approximate the physical properties of native tissue. Among the materials that have been used in bone tissue engineering, HAP and their derivatives, such as β-TCP, are the most common scaffold materials for osteogenic induction of MSCs. Bioresorbable β-TCP–based scaffolds, compared to HAP-based ones, showed comparable results on ectopic bone formation [333] and, moreover, both synthetic ceramics support osteogenic differentiation more efficaciously than demineralized bone matrix [334]. To improve the affinity of osteoconductive ceramics for cells, HAP has been coated with bioactive peptides [335] or proteins [336], to enhance MSC attachment and osteogenic differentiation. In addition, surface modifications of bone-inductive scaffolds have been developed, such as using cerium oxide nanoparticle [337,338] or miRNA [339] to bioactivate the scaffold interface, to promote bone formation by MSCs and enhance angiogenesis.

Composite scaffolds, composed of multiple materials, are expected to be physically and biologically superior to single material–based scaffolds, as the properties of a composite may be programmatically varied by mixing different materials in various ratios. Both the composition and the relative ratio of the constituent materials can affect bone formation. HA has been used as a primary material combined with other materials such as TCP [340], poly-lactic-*co*-glycolic acid (PLGA) [341], and chitin [342] to produce various composite scaffolds. It was reported that scaffolds with different ratios of HA/TCP loaded with MSCs showed different extents of bone formation in vivo. Composites in which the HA/TCP ratio was designed to coordinate scaffold degradation with tissue deposition seemed optimal in promoting the greatest ectopic bone formation [343].

Finally, growth factors, cytokines, and other nonproteinaceous chemical factors are critical for osteogenic differentiation of MSCs. To successfully augment bone formation, it is necessary to continuously introduce osteoinductive molecules, most of which have a short half-life, in a spatially and temporarily controlled manner into the cell culture or the defect site. One strategy for enhancing bone formation is to use biomaterial scaffolds both as a cell carrier and a reservoir for the release of growth factors in a controllable manner. BMP2 is the most efficacious growth factor among the BMP family members and has been incorporated in various forms of biomaterial scaffolds to induce osteogenesis in ex vivo cultures [344], or to stimulate bone formation in vivo [345], as well as in clinical applications (e.g., INFUSE Bone Graft/LT-CAGE Lumbar Tapered Fusion Device, Medtronic). For example, Zhao et al. employed microfluidic technology to encapsulate bone marrow–derived MSCs and growth factors within injectable, photocrosslinkable gelatin methacrylate (GelMA) microspheres, producing a combination of MSCs, BMP2, and scaffold as a unit construct, resulting in increased bone mineralization and osteogenesis [346]. Another approach recently developed in our laboratory is to combine MSCs and BMP2 gene-based therapy approaches, delivered in a photocrosslinkable hydrogel scaffold, to enhance bone formation in vivo [347–349].

More importantly, in view of the considerable self-healing ability of bone, namely, that bone defects have an inherent regenerative capability, the more challenging current clinical need is to enhance bone remodeling and regeneration under various chronic disease conditions, such as osteoporosis or osteonecrosis. The mechanisms and feasibility of tissue regeneration for these bone diseases are under intensive investigation. For example, the microenvironment of inflammation and related reactive oxygen species production have been shown as essential for the biological activities of tissue-engineered grafts, which can be regulated by chemical compounds [350–352]. Proinflammatory cytokines produced by host cells can affect the results of musculoskeletal tissue engineering, especially under specific pathological conditions such as tumor [353]. In MSC-based bone tissue engineering, osteoclasts from the myeloid lineage are also active participants, in processes mediated by small RNAs and hormones [354,355]. Recent work by Lai et al. showed a promising way to use a 3D printed PLGA/TCP scaffold in combination with the bioactive molecule, Icariin, that can increase bone regeneration under the challenging condition of osteonecrosis [356].

Osteochondral tissue engineering

Severe joint defects often extend to damage or destruction of subchondral bone, leading to associated pain and mechanical instability of the joint. Clinical results show that, even for a partial thickness cartilage defect, there are beneficial effects in exposing subchondral bone by drilling, as well as the incorporation of a bone layer as an anchor to securely integrate grafts with host tissue. Osteochondral grafts or plugs are used clinically in the treatment of both chondral and osteochondral defects. However, poor integration of allograft with host tissue leads to eventual graft failure. Although a variety of methods, such as pulse lavage, have been proposed to remove allogenic bone marrow to decrease immunogenicity and facilitate host–graft integration; the results have been less than promising and better techniques for processing osteochondral allograft plugs are required [357].

Tissue engineering offers a promising alternative to autologous osteochondral grafts through the combination of biocompatible materials possessing widely varying physical properties with the multilineage differentiation potential of MSCs. Chondrogenesis and osteogenesis of

MSCs require different biophysical and biochemical cues from matrices and soluble growth factors/cytokines, respectively. A commonly used approach is to fabricate cartilage and bone independently before integrating those using sutures or glues. MSCs loaded into a hyaluronan or a TCP ceramic scaffold were separately induced to undergo chondrogenic or osteogenic differentiation, respectively. Subsequently, these two components were integrated together with fibrin sealant, becoming a single unit of an osteochondral construct [358,359]. One drawback with this method is the less-than-optimal integration between the chondral and bone constructs; poor integration leads to uneven cell distribution at the interface and/or the possibility of eventual separation of the two components.

Several groups have reported different alternatives to improve interface integration. In one approach, MSCs after in vitro chondrogenic or osteogenic differentiation were loaded into two separate PEG hydrogel layers before the cell-loaded PEG gels were photopolymerized together. Since the two components polymerized together, the osteochondral construct exhibited a less defined gap-line [360]. Another approach was to apply a press-coating process [361] to fabricate osteochondral constructs. Specifically, a PLA scaffold was pressed into a high-density pellet of chondrogenically induced MSCs then seeded at the opposite end with osteogenically differentiated MSCs. Macroscopically, the osteochondral composite consisted of a cartilage-like layer adherent to, and overlying, a dense bone-like component [362]. Since both cartilage and bone were produced in a single unit, no gap developed between the two tissues; instead, an interface resembling the native osteochondral junction was observed. Recent attempts have included the combination of MSCs encapsulated in microbeads of collagen type I, separately induced into osteogenic and chondrogenic lineages, with an interface consisting of undifferentiated MSCs, which resulted in a trilaminar osteochondral construct with an interzonal "tidemark"-like structure reminiscent of native interfacial tissue [363].

The ideal scenario for the fabrication of biphasic osteochondral constructs would be to differentiate MSCs cultured in a single unit scaffold into chondrocytes on the top and osteoblasts on the bottom. To this end the biomaterial scaffold should chemically and structurally support both chondrogenesis and osteogenesis. Nanofibrous scaffolds morphologically resembling natural ECMs have been shown to successfully support both chondro- and osteo-genesis of MSCs in vitro [364,365]. In addition, for in vivo applications, growth factors such as SDF-1 may be incorporated into the scaffolds to facilitate local stem cell homing and tissue regeneration [366].

Another need for achieving this goal is a culture system in which an MSC-laden construct can differentiate into cartilage and bone simultaneously. A double-chamber bioreactor with a unique two-compartment design allowing the storage and delivery of different media was used to culture osteochondral constructs [326,367,368]. With this double-chamber bioreactor, it would be feasible to engineer an autologous osteochondral graft using MSC-embedded biomaterial scaffolds. In addition to producing an integrated, biphasic graft with osteochondral-like composition and architecture, an additional requirement is to achieve adequate load-bearing properties to restore joint function and movement.

Subchondral bone reconstruction is critical in providing supportive structure for the repair of osteochondral defects [369]. Given the native mechanically demanding requirements, there are few if any grafts that exhibit adequate tensile and compressive properties capable of withstanding the high loads in the articular joint. Recent advances in biomaterials have shown potential in addressing this issue with the advent of a 3D-printable, biohybrid scaffold comprising supramolecular hydrogen bonding strengthened hydrogel comprising methacrylated gelatin and poly(*N*-acryloyl 2-glycine) [370]. Recently, other photocrosslinkable, biocompatible polymeric scaffolds have also been used to fabricate constructs with excellent mechanical properties that are comparable to those of native cartilage, including poly-D,L-lactic acid/PEG/poly-D,L-lactic acid [123], which may be bioactivated with the incorporation of hyaluronic acid to enhance chondrogenesis [323]. Applications of such scaffolds that possess biomimetic and adequate mechanical properties are expected to achieve better clinical outcomes.

Engineering other skeletal tissues with mesenchymal stem cells

MSCs may also be applied for the repair of a number of other musculoskeletal tissues besides bone and cartilage. There have been studies on the use of MSCs and tissue specific stem/progenitor cells in tendon/ligament, meniscus, muscle, and nerve repair. MSCs derived from different tissues display generally similar phenotypes but also different phenotypic and differentiation capabilities, partly attributable to their shared developmental origins [371] as well as the natural presence of multipotent MSC variants within these target tissues [25,372] or from neighboring tissues [373].

Tendon/ligament

In tendon/ligament repair (see Fig. 49.4, overview of tendon structure), early studies showed that rabbit MSCs contract collagen type I carrier gels and that the delivery of these constructs to patellar and Achilles tendon defects improved the biomechanical properties of the repair tissue

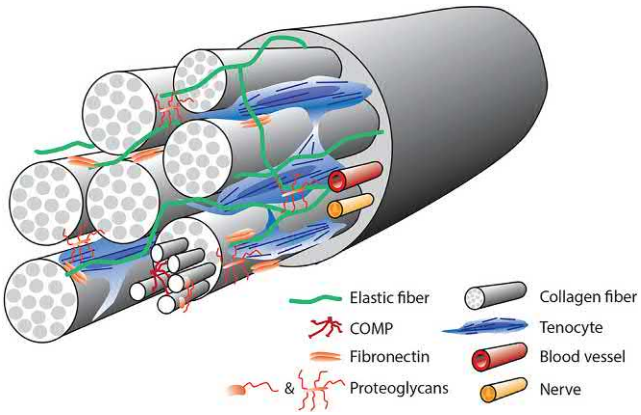

FIGURE 49.4 Overview of tendon tissue architecture. Tenocytes reside between collagen fibers and deposit extracellular matrix proteins into the microenvironment. The proteoglycans, decorin, biglycan, fibromodulin, and lumican, are involved in collagen fibrillogenesis and stem cell niche maintenance. Besides proteoglycans, other types of glycoproteins are also important constituents of tendon for cell adhesion and structural integrity, such as fibronectin, COMP, and lubricin. The collagen fibers are wrapped by a layer of connective tissue known as endotenon that contains blood vessels, lymphatics, and nerves. *COMP*, Cartilage oligomeric matrix protein. *Adapted with permission from [374] Rothrauff BB, Yang G, Tuan RS. Tendon resident cells—functions and features. In: Gomes ME, Reis RL, Rodrigues MT, editors. Tendon regeneration: Understanding tissue physiology and development to engineer functional substitutes. Academic Press (Elsevier), 2015. Chapter 2, pp. 41–76.*

as compared to acellular controls [375]. Newly emerging silk scaffolds that have been modified with RGD moieties and/or formed into nanofibrous scaffolds may also prove useful for tendon/ligament tissue engineering with MSCs [376]. Hybrid scaffolds consisting of silk and bFGF-releasing PLGA stimulated MSC proliferation and increased expression of tendon specific markers [377]. Using SDF-1 releasing collagen–silk scaffold, in combination with intra-articular injection of ligament-derived stem/progenitor cells, can enhance both anterior cruciate ligament (ACL) regeneration and bone–ligament–bone healing in rabbit models [378]. Studies applying tensional and torsional mechanical stimulations to MSC-seeded collagen gels in vitro have shown enhanced tenogenesis [379]. Adipose-derived MSCs have also recently been used for enthesis healing after the repair of acute and chronic massive rotator cuff tears in rats [380]. These studies suggest a potential role for MSCs in regenerative applications for tendon and ligament defects, particularly with the modulation of the mechanical loading environment. MSCs have also been applied to replicate the hierarchical, multitissue transition features in injured tendon/ligament tissues [381]. As tendon/ligament injuries are typically localized to the bone-tendon/ligament interface, there have been multiple reports on the fabrication of fibrous scaffolds with a mineral gradient to mimic features found in native entheseal tissue [382,383]. In such applications, MSCs have demonstrated promise for tendon/ligament repair, where fibrous scaffolds and mineral composition are found to induce adipose-derived MSC alignment and matrix mineralization, respectively, via topographical and biochemical cues [383]. Finally, an urea-solubilized extract of bovine Achilles tendon has recently been shown to exhibit strong protenogenic effect on human MSCs [384,385] and has been incorporated into a composite gelatin and electrospun-aligned PCL nanofibrous scaffold for tendon/ligament tissue engineering [386,387]. More details regarding cellular therapy in enthesis tissue repair can be found in a recent review article [381].

Meniscus

The meniscus is the most commonly injured structure in the human knee. To date, meniscus repair approaches has revolved around surgical techniques and development of meniscus tissue engineering. However, until recently, there have been limited options for partial replacement of meniscus. This is due in part to the fact that, for functional restoration of meniscus, the biomaterial scaffolds need to be mechanically robust to sustain weight bearing and movement, as well as having strong regenerative potential, given that the central portion of meniscus has a poor blood supply that impedes successful graft remodeling and healing [388]. Accordingly, exogenous MSC application has been used to the augment repair of the fibrocartilaginous knee meniscus. Multipotent MSC-like cells have been identified in the meniscus, with some zonal specificity [389]. It has been reported that intra-articular injection of MSCs derived from synovium [390] and meniscus-derived tissue specific stem/progenitor cells [391] (absent a tissue engineering delivery vehicle) can have an ameliorating effect on meniscus defect–induced joint degeneration. Recent studies combining MSCs with cartilage-promoting agents, such as growth factors and tissue-derived ECMs, have shown promising healing outcomes in meniscus repair [392–397]. It is noteworthy that, compared to classic growth factor-based approach, the use of tissue-derived ECM takes advantage of their natural, tissue- and region-specific bioactivity on progenitor cells [394,397]. Given that knee meniscus exhibit region-specific compositional and healing differences, an ECM-based approach is likely to bring more precise, efficacious treatment for meniscus repair. In addition to combining with various cartilage-promoting agents, MSCs are seeded on various types of scaffolds and implanted into meniscal defects. These scaffolds included devitalized meniscal allografts [398] fibrin glue [399], hyaluronan/gelatin [400] hyaluronan/collagen [401] composite matrices, and kartogenin with platelet-rich plasma gel [392].

A collagen-based meniscus implant (also known as Menaflex) has shown promising results in Europe and is available in the United States as the only approved collagen scaffold for segmental augmentation for the medial meniscus after overcoming regulatory hurdles [402]. Recent studies combining MSCs with cartilage-promoting agents, such as growth factors and tissue-derived ECMs, in combination with appropriate biomaterial scaffolds, have shown promising healing outcomes in meniscal repair [392–397]. Clearly, MSCs can play a role in the reparative process of the meniscus, although the mode of their administration and the nature of the carrier materials require further optimization.

Gene therapy in musculoskeletal tissue engineering

Gene therapy is a promising technique for disease treatment, in which genetically modified cells are transferred into or generated within individuals for therapeutic purpose. The cells with modified genes can be induced to differentiate into desired cell types or produce proteins needed for tissue repair. Although safety issues still remain, a likely future scenario is the combination of gene therapy, cell therapy, and tissue engineering for the treatment of musculoskeletal diseases.

There are two strategies that are currently used in gene therapy, viral transduction and nonviral transfection, and both strategies can be conducted in vivo and ex vivo [403]. In vivo gene therapy is simple but often lacks controlled tracking of the gene-modified cells and target site specificity. An attractive alternative is to combine gene therapy with tissue-engineering approaches to transduce or transfect cells of interest ex vivo and then use a carrier or scaffold to deliver these genetically modified cells to the target site. This approach offers the advantages of flexibility of target cell type and retainment of gene-modified cells at the site of interest. Gene transduction using viral vectors, such as retrovirus, adenovirus, and lentivirus, effectively modifies the host chromosome but raises concerns about mutagenesis and possible immune reactions. In contrast a nonviral approach is safer but the efficiency of transduction is lower. More research efforts are needed to overcome the limitations associated with each approach. The ultimate goal is to develop a simple, safe, and effective means to transfer genes into cells of interest.

Currently, the most frequently applied example of gene therapy in bone and cartilage tissue engineering is the transduction/transfection of MSCs with BMP genes. BMPs play important roles in the regulation of osteogenic differentiation of MSCs and the production of bone matrix during bone formation. To overcome the short half-life of growth factors, MSCs are transduced or transfected with BMP genes for continuous protein expression in vitro and in vivo. BMP2-transduced MSCs were induced to differentiate into osteoblasts producing bone matrix and synthesize BMP2, thus attracting host cells to migrate and differentiate. Compared to control MSCs, the BMP2-producing MSCs effectively enhance bone formation, even at large defect sites such as segmental femoral defects [404]. Similar results have also been reported when BMP2 or BMP4-transduced MSCs were delivered to bone defects using different biomaterial scaffolds, such as demineralized bone matrix [404], gelatin [405], β-TCP [406], and calcium phosphate cement [407]. Currently, several research groups are testing the effects of overexpressing a combination of factors such as BMP with VEGF, a potent inducer of angiogenesis [408]. The potential benefits of cartilage repair using gene therapy are also gaining recognition. For example, BMP7-transduced MSCs delivered by PGA scaffolds successfully regenerated cartilage, whereas the control nontransduced group did poorly [409], and BMP4 transduction induced chondrogenesis and enhanced cartilage repair [410]. In our recent work, we have successfully demonstrated the utility of activating an MSC-embedded PLLA scaffold with AAV-BMP2 gene construct, resulting in gene transduction of cells in situ and concomitant BMP2 production, to achieve enhanced osteogenesis in vitro and bone formation in vivo, which represents a new delivery strategy for combined gene and cell-based therapy [347–349].

Conclusion and future perspectives

Due to their ease of isolation, their capacity for undergoing in vitro proliferation to achieve a large number of cells for cell therapy, their ability to undergo lineage specific differentiation into musculoskeletal cells, and their potential immunomodulatory advantages, MSCs present significant potential in musculoskeletal skeletal tissue engineering, which promises to bring hope to patients and surgeons alike for the generation of functional tissue substitutes. As discussed in this chapter, MSCs have been used in tissue engineering of a number of musculoskeletal tissues, including cartilage, bone, osteochondral constructs, ligament, and tendon. Currently, tissue-engineered constructs and biologics have not been readily accepted for clinical use to treat musculoskeletal tissue defects [411,412]. There clearly is a need for further research that combines concerted efforts of biologists, engineers, and clinicians. Critical to the success of these approaches is a better understanding of MSC biology. So far, there has not been a marker that can be used to unequivocally identify and prospectively select MSCs from diverse tissues. The various growth factors, signaling pathways, and transcription factors that regulate phenotype transition, that is,

uncommitted versus differentiated phenotype, remain to be identified and studied. The ultimate fate of the implanted cells also needs to be ascertained. In addition, the microenvironmental conditions that optimally allow MSCs to differentiate completely and stably into a desirable lineage require further elucidation. Despite our incomplete understanding of MSC biology, their use in musculoskeletal tissue engineering is of high potential. Current and future efforts in this area should focus on the fabrication of optimized scaffolds, systematic studies of the interplay of MSCs, scaffold and environmental factors, and the development of quantitative outcome measurements, both in vitro and in vivo, for tissue-engineered constructs. Ultimately, in vivo, functional testing serves as the gold standard to assess the long-term utility of the engineered constructs and warrants much greater attention [412]. The immunosuppressive and antiinflammatory effects of MSCs present a potentially powerful biological target for their use in allogeneic transplantation in the inflammatory environment of an injury site. With further research and development the use of MSCs in tissue engineering is expected to bring to fruition the generation of functional tissue substitutes, either in situ or suitable for implantation to improve the quality of life of patients with debilitating musculoskeletal injuries.

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Chapter 50

Bone tissue engineering and bone regeneration

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Introduction

Medical advances have led to a welcome increase in life expectancy. Indeed, by 2020, 20% of the population will be over 65. However, this progress presents its own new challenges: increases in age-related diseases, associated reductions in quality of life, and attendant substantial socioeconomic cost [1]. Fractures alone cost the European economy €17 billion and the US economy $20 billion annually. In the United States, there are some 8 million bone fractures per year, of which approximately 5%–10% are associated with delayed healing or nonunion. Furthermore, it is predicted that the number of hip fractures worldwide will increase from 1.7 million in 1990 to 6.3 million in 2050 emphasizing the importance of enhanced knowledge around skeletal reparative approaches for bone. Thus there is now an urgent unmet need to develop robust regenerative strategies to enhance bone loss and improve quality of life. Recent advances in the isolation of the human skeletal stem cell confirm the skeletal stem cell confer to bone its innate capacity for regeneration, repair, and remodeling in response to mechanical stimuli and regeneration upon damage providing, alongside skeletal development, a natural paradigm informing tissue engineering strategies. Bone regeneration strategies seek to harness and enhance this reparative capacity for skeletal tissue replacement lost as a consequence of aging, disease, or trauma. Critical in the development of approaches to repair skeletal tissue is an understanding of the skeletal cell, skeletal cell function, growth factor interaction, and the appropriate development of biomimetic scaffolds that incorporate/deliver growth factors/biological cues together with mechanical cues to aid formation (Fig. 50.1).

Bone tissue engineering could potentially offer an effective, personalized therapeutic option with a reduced risk of disease transmission, infection and immunogenicity, and limitless availability. Although the complex processes that drive and regulate the reparative capacity of bone have yet to be fully elucidated, current approaches seek to facilitate bone reparation by providing a suitably conducive microenvironment that does not interfere with, while synergistically accelerates, native regenerative processes. While this rationale may appear simple, it has proven to be extremely difficult to execute, evidenced by limited clinical translation. This review details current development in skeletal cells, scaffold development, and preclinical models for bone tissue formation. Finally, we review current regenerative strategies to augment bone formation in a range of orthopedic applications and the challenges and opportunities that present.

Skeletal stem cells

Adult bone marrow (BM) contains a small population of nonhematopoietic stroma–derived stem cells, which exhibit extensive proliferation and multilineage differentiation potential. These multipotent progenitor cells referred to as skeletal stem cells, or more commonly mesenchymal stem cells (MSCs), are capable of differentiating into osteoblasts, chondrocytes, and adipocytes (reviewed in Ref. [1,2]). Regenerative strategies for skeletal tissue seek to harness and enhance the reparative capacity of the skeletal stem cell for skeletal tissue replacement damaged or lost through congenital defects, trauma, and as a consequence of aging. The multipotent, self-renewing stem, and progenitor stem cell of BM stroma are widely

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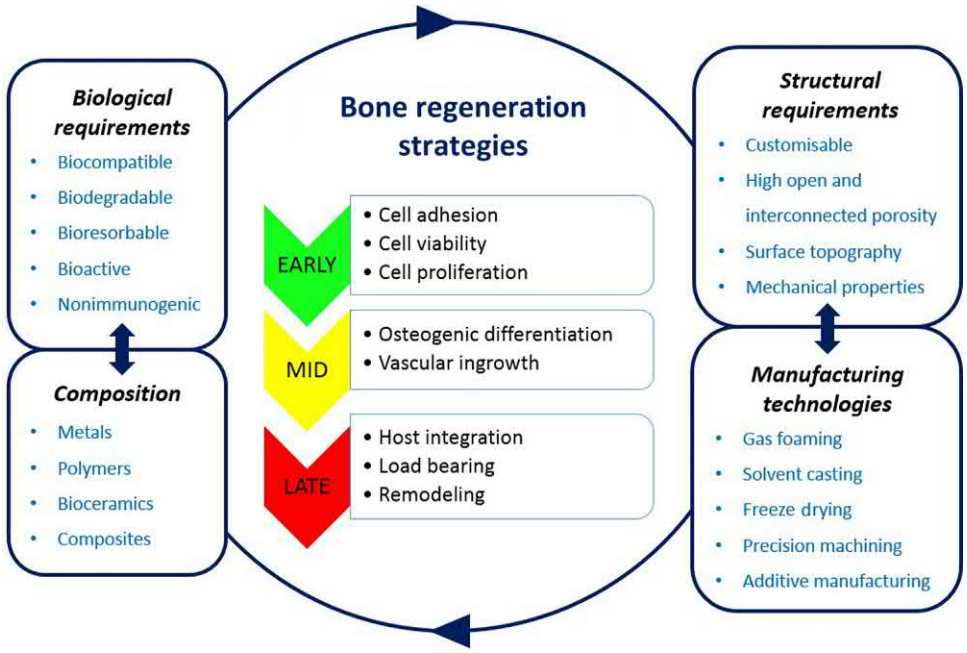

FIGURE 50.1 Interlinked factors in contemporary bone regeneration.

referred to as MSCs (a term originally coined by Caplan in 1991 and extensively used in the literature), mesenchymal stromal cell, as well as medicinal signaling cell, indicating the degree of uncertainty over the in vivo identity, phenotype, function, and clinical utility of cells typically designated as MSCs. Critically, "skeletal stem cells" was introduced by Bianco and Robey to denote, specifically, MSCs from BM stromal tissue that confers to bone its innate capacity for regeneration, repair, and remodeling (reviewed in Refs. [1–3]). The International Society for Cell Therapy proposed a minimal definition for multipotent "mesenchymal stromal cells" as (1) CD73, CD90, and CD150 positive; (2) CD11b, CD14, CD19, CD34, CD45, and HLA-DR negative; and (3) possess the capacity to differentiate into osteoblasts, chondrocytes, and adipocytes under in vitro stimulation [4]. However, in vitro assays of differentiation potential are themselves poorly predictive of in vivo function, either following transplantation or, more critically, in native tissues during turnover, and following injury. Seminal work by Alexander Friedenstein confirmed the presence of a bone-forming stem cell (1–10 per 100,000 nucleated cells), in the form of fibroblast-like clonogenic cells [colony-forming unit-fibroblastic (CFU-F)] tissue culture plastic adherent, non-hematopoietic fraction in BM. The progeny from the CFU-F generated bone and cartilage tissue following transplantation in diffusion chambers and, critically, a full ectopic BM organ when implanted on an open calcium phosphate scaffold [5,6] (reviewed in Ref. [1]). More recently, lineage tracing studies in mice have informed our knowledge on the role and potential of the skeletal stem cell for bone regeneration, with indication of a hierarchy of skeletal progenitors with defined surface phenotypes important in bone turnover and repair [7,8]. Thus Chan et al. demonstrated using ectopic transplantation assays, a defined lineage profile of skeletal stem and progenitor cells with osteo-, chondral-, and stromal-potentialities characterized by variation in expression of CD200, CD105, Thy, and 5C3 surface markers. Importantly, a similar developmental hierarchy of skeletal progenitors was also confirmed by the same authors to be present in humans [9]. Single-cell transcriptome analysis of the growth plate and diaphysis of human fetal femurs indicated that differential expression of a number of markers (PDPN, CD146, CD73, and CD164) on tissue formation evaluation form clonal populations revealed a developmental hierarchy of skeletal stem and skeletal progenitors. Chan et al. demonstrated that SSCs (Skeletal Stem Cells) ($PDPN^+$ $CD146^-$ $CD73^+$ $CD164^+$) gave rise to an early bone, cartilage, and stromal progenitor ($PDPN^+$ $CD146^+$), which in turn transitioned into osteo/stromal progenitors ($PDPN^-$ $CD146^+$) or chondroprogenitors ($PDPN^+$ $CD146^-$ $CD73/CD164^-$) prior to any tissue-specific terminal differentiation although these cells do not differentiate into adipocytes [9].

These rigorous in vivo studies have important implications for bone regeneration and bone fracture repair, providing evidence for the existence of spatially and temporally discrete pools of skeletal stem/progenitor populations within bone, involved in development and holding distinct physiological roles. Indeed, the recent identification of a $CD200^+$ $CD105^-$ periosteal stem cell

present in the long bones and calvarium of mice [10,11] distinct from the endosteal skeletal stem cell provides further insight into the repertoire of discrete skeletal progenitor pools. The authors observed periosteal stem cells displayed self-renewal and the ability to give rise to a hierarchy of progeny—including cartilage following injury and the potential to form bone ossicles following subrenal transplantation via direct intramembranous bone formation in the absence of cartilage formation or hematopoietic cell recruitment. Harnessing discrete appropriate skeletal cohorts has important implications for not only our understanding of bone function but the harnessing of appropriate skeletal fractions for intramembranous and endochondral bone repair.

The complexity that resides in dissecting out and understanding the requisite skeletal cohorts to employ in reparative bone strategies is perhaps reflected in the ongoing challenge of identifying a therapeutic effect following translational MSC transplantation studies with MSC grafting and a focus on paracrine or immunomodulatory mechanisms (independent of stem cell or tissue progenitor function). A wealth of data has emerged on the pleotropic effects of SSCs on cells of the immune system [12]. The phenotype of SSCs has been widely described as Major Histocompatibility Complex (MHC) Class I+, MHC Class II−, CD40−, CD80−, and CD86−. Although MHC Class I molecules have the potential to activate alloreactive T cells, the absence of CD80 and CD86, responsible for providing costimulatory signals necessary for T-cell activation and survival, would leave the T cells anergic. Macrophages are key players of the innate immune system in initiating and controlling inflammation, and SSCs can influence macrophage function depending upon the inflammatory context. Monocytes arriving at an inflammatory environment can develop into activated macrophages, which produce proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ). The resulting proinflammatory microenvironment activates SSCs to adopt an immunesuppressive phenotype and release mediators, which skew the differentiation of monocytes toward antiinflammatory macrophages that secrete high levels of interleukin (IL)-10, transforming growth factor-β1, and low levels of cytokines (TNF-α, IFN-γ, IL-1, IL-6), and aid tissue regeneration following inflammation. The regulatory effects of SSCs on immune and inflammatory cells will necessitate defined rigorous in vivo systems on the immunogenicity and immunomodulatory capacity of SSCs in specific pathophysiologic models. This will inform the full therapeutic potential of these cells.

Given osteoblasts are needed to make bone, the cell source employed in the cell reparative paradigm is as crucial as the choice of biomaterial (see Table 50.2). Incorporation of skeletal populations into bone tissue engineering has been a key advancement. BM-derived stem cells are the most frequently utilized stem cell source, given the wealth of information available and their ease of acquisition [1,3]. Adipose tissue-derived stem cells have been proposed as a viable alternative, given their reported osteogenic ability in vitro, ease of acquisition, abundance, and ability to survive low oxygen or glucose environments [13]. Such resilience is advantageous, particularly when the blood supply is limited. Recently, there has been an interest in using oral cavity MSCs and induced human pluripotent stem cells in bone tissue engineering [13,14] (Table 50.1).

Fracture repair—the (limited) self-reparative capacity of bone

Under optimal conditions, bone can heal completely without the formation of a fibrous scar, such that the regenerated tissue is indistinguishable from its state prior to injury [16]. Impairment in this remodeling process occurs in osteoporosis, which, in turn, results in increased fracture risk, particularly amongst the elderly. Fracture healing is a complex biological process that is intertwined with the innate immune system. Following trauma or damage, bone heals either by the direct intramembranous or indirect pathway. The latter is the most common and comprises of intramembranous and endochondral ossification. Primary fracture healing requires anatomical reduction and rigidly stable conditions, achieved by surgical intervention via open reduction and internal fixation methods, which minimizes the fracture gap and interfragmentary motion. In such conditions, bone is able to heal by direct regeneration of anatomical lamellar bone followed by Haversian systems, without the need for remodeling to occur. When a slightly larger gap exists between bone fragments, gap healing occurs, whereby the voids are filled with direct deposition of intramembranous woven bone. Haversian systems are reestablished through osteoclast-mediated remodeling [17,18]. However, complete rigidity is not possible in most fractures treated by splinting, intramedullary nailing, or external fixation methods. In these scenarios, secondary fracture healing, which involves intramembranous and endochondral ossification, occurs. In fracture repair the fibrin network provides the initial matrix for inflammatory cell influx triggered by platelet-derived factors, local tissue macrophages, complement fragments, as well as signals released from necrotic tissue. Neutrophils arrive within the first 24 hours and recruit macrophages and monocytes to the fracture site which, in turn, work together with macrophages resident in the peri- and endosteum to regulate the inflammatory response, a critical stage of secondary fracture healing [18]. Macrophages phagocytose necrotic cells

TABLE 50.1 Cell types used in bone tissue engineering.

Cell type	Multipotent or differentiated	Potential for bone tissue engineering	Advantages	Disadvantages
Bone marrow–derived stem cells	Multipotent	Osteogenic Potential for neovascularization	Relatively easy acquisition Extensively characterized	Donor morbidity Limited proliferative potential Fewer cells compared to other sources Dependent on age and health of donor
Umbilical vein stem cells	Multipotent	Osteogenic	High proliferation Minimal donor morbidity	Not extensively characterized
Oral cavity MSCs (dental pulp, gingival stem cells)	Multipotent	Osteogenic	Abundant Easy acquisition	Not extensively characterized
Adipose-derived stem cells	Multipotent	Osteogenic Potential for neovascularization	Easy acquisition Extensively characterized Able to grow in nonideal conditions	Donor morbidity (due to anesthesia)
EPCs, specifically ECFC	Lineage directed	Potential for neovascularization Supports osteogenic differentiation	Easy acquisition (peripheral blood, umbilical cord blood) Abundant Minimal donor morbidity Can be coseeded with bone marrow–derived stem cells	Not multipotent Limited proliferating potential of early EPCs Differences in isolation and cultivation procedures make comparison studies on EPC functionality difficult Requires coseeded cells for stabilization for neovascularization
Human umbilical vein endothelial cells	Differentiated	Potential for neovascularization	Easy acquisition Can be coseeded with bone marrow–derived stem cells	Not multipotent
Induced human pluripotent stem cells	Multipotent Differentiated	Osteogenic Chondrogenic	Easy acquisition Minimal donor morbidity Patient specific Unlimited self-renewal and higher proliferative capacity than MSCs Relatively established cell reprograming protocols produce lineage-specific cell types from any cell source	Variable cell reprograming efficiency. Protocol optimization still needed Necessary induction into high-quality progenitor cells posttransplantation Risk of tumor formation

ECFC, Endothelial colony-forming cells; *EPCs*, endothelial progenitor cells; *MSCs*, mesenchymal stem cells.

Source: Adapted from [13] Szpalski C, Barbaro M, Sagebin F, Warren S. Bone tissue engineering: current strategies and techniques—Part II: Cell types. Tissue Eng, B: Rev 2012;18(4):258–69; [14] Csobonyeiova M, Polak S, Zamborsky R, Danisovic L. iPS cell technologies and their prospect for bone regeneration and disease modeling: a mini review. J Adv Res 2017;8(4):321–7; Liu Y, Chan J, Teoh S. Review of vascularised bone tissue-engineering strategies with a focus on co-culture systems. J Tissue Eng Regen Med 2015;9(2):85–105 [15].

and the initial fibrin mesh and secrete chemotactic and inflammatory mediators such as TNF-α, IL-1β, IL-6, and C–C motif chemokine ligand 2, which trigger MSC, local osteoprogenitor cell, and fibroblast recruitment. Stromal cell–derived factor 1 mediates the local and systemic recruitment of MSC, while platelet- and macrophage-derived signals guide the proliferation, differentiation, and extracellular matrix (ECM) synthesis of recruited MSC and osteoprogenitor cells. This enables the removal of the hematoma and cessation of the acute inflammatory response within a week after the initial damage, allowing for the generation of granulation tissue rich in

TABLE 50.2 Comparison of contemporary bioengineered bone scaffold types.

Scaffold type	Advantages	Limitations
Metals (or metal alloys), e.g., titanium, cobalt, nickel, tantalum, stainless steel	Biocompatible Superior mechanical properties which could be useful in situations of slow bone (in)growth Bioactivity can be enhanced by surface modification techniques Established fabrication techniques and already in clinical use	Expensive Stress-shielding Poor biodegradability Potential ion release may cause local and distal toxicities Manufacturing processes unsuitable for biofabrication use
Hydrogels, e.g., collagen, gelatin, alginate, hyaluronic acid, fibrin, poly(ethylene oxide), poly(ethylene glycol)	Generally biocompatible High water content allows for cell encapsulation and growth Controllable mechanical properties and biodegradability Tunable biomolecule release 3D patterning to mimic tissue microarchitecture(s) Multimaterial compositions	Limited physical manipulation Limited mechanical strength. Increasing gel mechanical properties can reduce cell survival and functionality Random cell distribution Time-consuming optimizations required, particularly for bioprinting applications Bioactivity dependent on gel composition and properties
Polymers • Natural, e.g., alginate, gelatin, chitosan, silk • Synthetic, e.g., polycaprolactone, poly-L-(lactic acid), poly(lactic-*co*-glycolic acid)	Low cost Biocompatible Tunable biodegradability Mechanical properties and bioactivity can be controlled by adjusting compositions	Limited mechanical properties Can lack bioactivity Performance and purity affected by manufacturing quality Time-consuming optimizations required, particularly for bioprinting applications, and composites
Bioceramics, e.g., tricalcium phosphate, (nano)hydroxyapatite, biphasic calcium phosphate	Osteoinductive and osteoconductive properties enhance osseointegration Biocompatible, similar to native bone mineral content Ease of manufacturing and delivery, e.g., paste, injectable, bioprintable Low cost	Inherent brittleness limits its mechanical property when used in isolation Uncontrolled degradation and resorption in vivo Unsuitable for cell encapsulation when used in isolation
Bioactive glass, e.g., Bioglass 45S5	Osteoconductive properties enhance osseointegration Biocompatible Tunable bioactivity Already in clinical use	Inherent brittleness limits its mechanical property when used in isolation Limited physical manipulation Poor control of resorption Potential release of toxic ions

Source: Modified from [29] Turnbull G, Clarke J, Picard F, Riches P, Jia L, Han F, et al. 3D bioactive composite scaffolds for bone tissue engineering. Bioact Mater 2018;3(3):278–314.

proliferating mesenchymal cells and neovasculature embedded within a collagen matrix [17,18].

Disruption to local vasculature with subsequent reactive arteriolar contraction results in a hypoxic environment, particularly in the area adjacent to the fracture gap. This low oxygen tension, together with a degree of micromotion and microenvironmental signals, guide MSC differentiation along the chondrogenic pathway. Chondrocytes produce cartilage, which extends throughout the gap, connecting the ends of the fracture. In conjunction with fibrotic tissues the soft callus provides initial mechanical stability and becomes the scaffold for endochondral bone formation. While this occurs, in areas with improved blood supply and greater stability, new woven bone forms via intramembranous ossification. This begins in the inner layer of the periosteum and advances toward the gap. The woven bone covers the external surface of the soft callus. Chondrocytes in the soft callus hypertrophy and undergo apoptosis, secreting calcium and factors that stimulate vascular ingrowth into the cartilage scaffold-stabilized gap. Increased blood flow is accompanied by osteoprogenitor cell differentiation into osteoblasts and deposition of woven bone onto the cartilage scaffold, creating a hard callus. As the cartilage mineralizes, the mechanical stability of the fracture site increases until the new bone formed is able to support mechanical loads independently. Osteoclasts remove immature woven bone and cartilage matrix, initiating remodeling, which

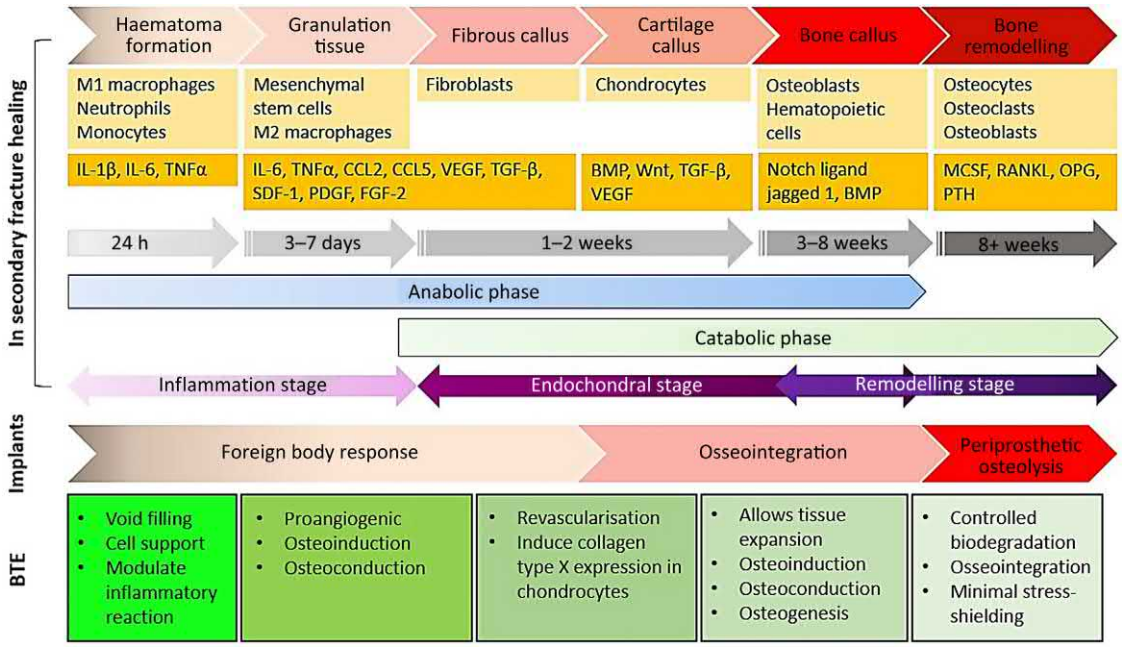

FIGURE 50.2 Stages of secondary fracture healing, compared to bone healing with implants and the key properties of bioengineered constructs that could support each stage.

BMP, Bone morphogenetic protein; *BTE*, bone tissue engineering; *CCL*, C-C motif chemokine ligand; *FGF-2*, fibroblast growth factor 2; *IL-1β*, interleukin-1β; *IL-6*, interleukin-6; *MCSF*, macrophage colony-stimulating factor; *OPG*, osteoprotegerin; *PDGF*, platelet-derived endothelial growth factor; *PTH*, parathyroid hormone; *RANKL*, receptor activator of nuclear factor kappa-B ligand; *SDF-1*, stromal cell−derived factor 1; *TGF-β*, transforming growth factor-β; *TNF-α*, tumor necrosis factor alpha; *VEGF*, vascular endothelial growth factor; *Wnt*, wingless-related integration site. *Adapted from [17] Einhorn T, Gerstenfeld L. Fracture healing: mechanisms and interventions. Nat Rev Rheumatol 2015;11(1):45−54; [18] Loi F, Cordova L, Pajarinen J, Lin T, Yao Z, Goodman S. Inflammation, fracture and bone repair. Bone 2016;86:119−30; [21] Winkler T, Sass FA, Duda GN, Schmidt-Bleek K. A review of biomaterials in bone defect healing, remaining shortcomings and future opportunities for bone tissue engineering: the unsolved challenge. Bone Joint Res 2018;7(3):232−43.*

regenerates the Haversian system. However, the establishment of the normal integrity of bone can take several months to complete [18].

Around 5%−10% of all bone fractures are prone to delayed bone union or progress to a nonunion or pseudoarthrosis [19]. Successful fracture healing is thus dependent on interlinking factors, which affect the mechanical stability of the fracture, the influx of osteogenic and inflammatory cells, growth factors and chemotactic mediators, and adequate vasculature. Underlying pathological conditions and factors such as aging, smoking, diabetes mellitus, and obesity can adversely affect bone healing as well as aging [18,19]. Current therapeutic approaches involve artificial implants that last 15−20 years. Revision surgery in patients who are less medically fit can be fatal. Postoperatively, patients may face the prospect of losing their independence or living with a disability, through loss of function or mobility. These patients might additionally have to endure chronic pain caused by a malfunctioning implant, which could worsen any underlying comorbidities or result in anxiety and/or depression. Given bone is the second most transplanted tissue [20], and increasing aging demographic, there has never been a greater need for new bone reparation approaches (Fig. 50.2).

A framework for bone repair: biomaterial-driven strategies for bone regeneration

Bone tissue engineering scaffolds typically consist of a solid support structure possessing an open and interconnected pore network, while matrices are often hydrogel based. Both forms must possess appropriate biophysicochemical properties (such as mechanical strength, stiffness, biodegradability, and surface chemistry) for tissue formation, as well as having the capability to withstand and respond to mechanical stresses (Fig. 50.1) [22]. In silico and multimodal imaging and tracking approaches are being developed to help identify optimal scaffold design parameters for bone [23−25]. Conventional methods used to fabricate bone tissue engineering scaffolds have (typically) variable abilities in controlling pore size,

geometry and interconnectivity, and upscalability (Fig. 50.1) [26]. Therefore scaffold design and manufacturing techniques are crucial factors that determine the biomechanical efficacy of the scaffold in vivo. These factors are in turn dependent on the properties of the biomaterial(s) to be used. Scaffolds typically consist of a biomaterial made of bioceramics, metal, self-assembly peptides, synthetic, or natural polymers [27,28]. Composite scaffolds are increasingly used to overcome the specific advantages and disadvantages of each type of biomaterial. Several combinations of biomaterials, with and without cells, have been investigated using in vitro and/or in vivo methods for their potential use in bone [20,29].

Different surface modification techniques, such as surface coating, electrochemical deposition, oxidization, or anodization via cathodic pretreatment, can be employed to further enhance the biocompatibility and osteoinductive capability of biomaterials [30]. These postmanufacturing processes alter surface roughness (and thereby, topography) and/or the wettability of the biomaterial(s). However, despite in vitro successes, the number of in vivo studies showing enhanced osseointegration is limited [30]. This is reflected by the paucity of biomaterials that have achieved clinical translation, and with limited efficacy [27]. There remains no clear consensus as to which biomaterial, or combination thereof, scaffold design or manufacturing approach, are optimal for bone regeneration (Table 50.3).

Growth factors: biomimetic-driven strategies for bone regeneration

In vivo, biochemical signals in the form of growth factors, hormones, and/or cytokines are secreted at local injury sites or in areas undergoing bone remodeling, triggering the migration of progenitors and inflammatory cells (as in the case of bony injury) or the activation of osteoblasts and osteoclasts (as with bone remodeling) [31,32]. This allows for the generation of new bone tissue as part of the healing or remodeling process respectively. Inadequate in vivo osseointegration of cell-free scaffolds has seen an increasing trend in the past decade toward the integration of cells and bioactive molecules into bone tissue engineering approaches, through harnessing their innate regenerative properties by the induction of physiological processes These integrated, biomimetic scaffolds have been termed as "smart scaffolds." Recent studies have begun to investigate the efficacy of synthetic peptides rather than growth factors (Table 50.4) [33], as well as the paracrine effect of the secretome of MSC or stromal cells (shuttled

TABLE 50.3 Growth factors currently investigated for bone tissue engineering.

Growth factor	Tissues studied	Observed function
BMP (-2, -7)	Bone, cartilage	Differentiation and migration of osteoblasts, with accelerated bone healing observed
FGF (-1, -2, -18)	Bone, muscle, blood vessel	Migration, proliferation and survival of endothelial cells Increased osteogenic differentiation of MSCs
IGF-1	Bone, cartilage, muscle	Proliferation and differentiation of osteoprogenitor cells
PDGF (-AA, -BB)	Bone, cartilage, blood vessel, muscle	Proliferation, migration, growth of endothelial cells Osteoblast replication in vitro Type 1 collagen synthesis
PTH	Bone	Increased bone formation through osteoblast stimulation with intermittent dosage. Bone resorption if administered continuously
TGF-β3	Bone, cartilage	Proliferation and differentiation of bone-forming cells Enhances hyaline cartilage formation in vivo Antiproliferative to epithelial cells
VEGF	Bone, blood vessel	Enhanced vasculogenesis and angiogenesis but functionality of vasculature dependent on concentration used Reduction or increment in bone formation dependent on concentration when used in combinational delivery with BMP-2

BMP, Bone morphogenetic protein; *FGF*, fibroblast growth factor; *MSCs*, mesenchymal stem cells; *PDGF*, platelet-derived endothelial growth factor; *PTH*, parathyroid hormone; *TGF*, transforming growth factor; *VEGF*, vascular endothelial growth factor.

Source: Reproduced from [24] Tang D, Tare R, Yang LY, Williams D, Ou KL, Oreffo R. Biofabrication of bone tissue: approaches, challenges and translation for bone regeneration. Biomaterials 2016;83:363–82.

TABLE 50.4 Synthetic peptides potentially useful as alternative agents for enhancing cell adhesion and osteogenesis.

Synthetic peptide sequence	Equivalent molecule
REDRV, LDV	Fibronectin
DGEA, GFOGER, ^{766}GTPGPQGIAGQRGVV^{780}	Collagen type I
GLRSKSKKFRRPDIQYPDATDEDITSHM	Osteopontin
FHRRIKA	Bone sialoprotein
KIPKASSVPTELSAISTLYL	BMP-2
^{105}YKRSRYT^{111}, ^{119}KRTGQYKLGSKTGPGQK^{135}	FGF-2
YIGSR, IKVAV	Laminin
RGD	Integrin-binding proteins

BMP, Bone morphogenetic protein; *FGF*, fibroblast growth factor.
Source: Adapted from [33] Kesireddy V, Kasper F. Approaches for building bioactive elements into synthetic scaffolds for bone tissue engineering. J Mater Chem B 2016;4(42):6773–86.

through extracellular vesicles or embedded within ECM molecules) to target osteoinduction, as well as the inflammatory and immunity processes involved in regeneration [34,35].

Different types of biomolecules need different delivery systems to achieve optimal therapeutic effects (Table 50.5 [34]). However, clinical studies typically employ growth factors at supraphysiological doses (mg/mL rather than ng/mL), resulting in adverse effects that include ectopic bone formation, antibody development, and possibly, carcinogenesis [28]. As such, only a few synthetic grafts containing bioactive molecules are commercially available for surgical use in certain countries [27]. There is no consensus as to the appropriate doses of growth factors or biomolecule combinations for bone tissue repair, with a wide range in use [36]. Furthermore, the actual dose of growth factors delivered by constructs in vivo when used in defects remains uncertain [37].

Bone biofabrication

In the past decade, additive manufacturing (AM) techniques have been used to manufacture 3D artificial implants using existing biomaterials in a precise and reproducible manner that is capable of meeting stringent performance criteria for clinical use [30,38]. The utilization of AM in the fabrication of bone tissue engineering constructs has also driven advancements in AM itself [24]. The incorporation of cells into the AM process to create integrated cell–biomaterial–biomolecule constructs is a recent development termed as biofabrication. Such an approach is typically divided into two categories based on the workflow pattern: top-down or bottom-up approach. Table 50.6 summarizes the three most commonly investigated 3D printing methods used for bone biofabrication. As bone tissue requires its composite cells to be at different stages of proliferation, differentiation, and maturation within a multilayered hierarchical structure, the ability to seed cells and biomolecules in 3D space, with a high degree of precision, in a user-controlled manner affords biofabrication an advantage over conventional techniques [24]. AM has been used for the concomitant spatial printing of different cell types on 3D scaffolds to generate complex tissues [39,40]. Another advantage of AM is the ability to incorporate biomolecules within a cellular matrix or within the scaffold itself during the printing process, providing another method for controlled drug delivery and gradient release [41,42]. Central to this approach remains the ability of printed cells to retain their functionality, as well as permitting bone remodeling by external and internal stimuli [43].

Biofabrication enables the generation of a customized 3D construct that is a closer fit to the defect (in comparison to traditional bone grafts), reducing the risk of engraftment or repair failure. Biofabricated bone will, ultimately, eliminate the need for donor bone grafts, allowing patients to have their operations earlier (thereby reducing wait times for surgery while regaining functionality sooner), and with lower physical and psychological morbidity. The risk of rejection of the biofabricated bone tissue is lower with the use of autologous cells compared to allogenic cells or graft. Thus biofabricated bone represents a potentially cost-effective way to treat patients with musculoskeletal defects, in addition to providing a new therapy for patients unable to be treated by conventional means. However, biofabrication is not without its limitations. The choice of biomaterial for biofabrication is currently limited by the AM method employed. AM

TABLE 50.5 Current delivery strategies for biomolecules in bone tissue engineering.

Biomolecule type	Delivery strategy
Nucleic acids, e.g., miRNA, siRNA	Intracellular and intranuclear preprograming
Small molecules, e.g., bisphosphonates	Surface adsorption, sustained release
Growth factor, e.g., BMP-2, VEGF	Surface presentation, controlled release
Endocrine, e.g., PTH, vitamin D_3, estradiol	Time-controlled release
Inorganic biomaterials, e.g., bioceramics, bioglass	Surface adsorption, surface deposition

BMP, Bone morphogenetic protein; *PTH*, parathyroid hormone; *VEGF*, vascular endothelial growth factor.
Source: Adapted from [34] Dang M, Saunders L, Niu X, Fan Y, Ma PX. Biomimetic delivery of signals for bone tissue engineering. Bone Res 2018;6:25.

TABLE 50.6 Advantages and limitations of common 3D printing methods used in bone biofabrication.

3D printing method	Advantages	Limitations
Microextrusion deposition	Good precision and microscale resolution Wide variety of biomaterials Capable of printing physical or compositional gradients, cell and bioactive factor bioprinting Potential for upscalable construct fabrication	Cell survival postbioprinting dependent on bioink properties, printing temperature, and build time Often requires use of support materials to fabricate porous constructs, increasing build time Low-to-moderate cost
Laser-assisted bioprinting	Nanoscale precision and prints at ambient conditions Capable of printing multiple physical or compositional gradients, and simultaneous cell and bioactive factor bioprinting	Remains extremely cost-prohibitive Limited upscalability
Inkjet-based cell printing	Low cost Capable of printing gradients and simultaneous cell and bioactive factor bioprinting, with high cell viability postbioprinting Relatively rapid fabrication	Limited biomaterial choice due to bioink printing requirements Poor resolution Limited upscalability

methods, such as stereolithography, require postprocessing procedures that are cytotoxic, while laser sintering can cause thermodegradation of the biomaterial, resulting in a loss of precise microstructure, which in turn affects material porosity and cell viability [38,44]. This limits the type of AM that can be employed for bone biofabrication purposes. No single biofabrication approach has yet been found to be ideal for bone tissue engineering purposes, with varying process protocols, limited upscalability, and questionable cell viability postbioprinting thus far limiting progress in the field [24].

Development of vascular bone

Bone is a metabolically active tissue supplied by an intraosseous vasculature with osteocytes located no greater than 100 μm from an intact capillary [45]. Thus a key issue to be addressed is the generation of a vascularized construct. Critically, complex engineered 3D constructs of clinically relevant size cannot be sustained by diffusion alone, and the development of a functional vascular network is necessary for ensuring a nutrient supply and equally important, waste removal, throughout the construct [46]. The integrated blood vessels within the bone matrix act as a regulator of bone integrity with the provision of stem cells, growth factors, and spatiotemporal cues [47]. This is the mainstay of the developing femur, particularly in endochondral bone formation, whereby incursion of blood vessels modulate and control the bone tissue to develop in a coordinated and systematic manner. Recapitulating these series of events will provide skeletal engineers with the tools to address the clinical problems of replacing and regenerating large bone defects. At the molecular and cellular level, the cross communication between vascular cells and skeletal cells (whether they be stem or differentiated osteoblasts) is

significantly effective in directing the cells toward skeletal patterning. Factors that regulate angiogenesis also play a role in bone development and repair. In fracture repair vascular endothelial growth factor (VEGF) activity is fundamental to angiogenesis, stem cell recruitment, callus formation, and endochondral ossification [47]. This tight coupling of osteogenesis and angiogenesis has moderated the direction of tissue engineers to develop models, materials that can mimic both in vitro and in vivo these interactions to develop better strategies to improve nonunion fracture repair.

Functionalization of biomimetic materials whether they be ceramics, polymers, or hydrogels to release angiogenic inducing factors such as VEGF has shown improved bone repair and regeneration [48]. Composite scaffolds designed to release VEGF and bone morphogenetic protein (BMP) 2 via different dynamic release profiles improved bone healing and vascularization when implanted in critical-sized skeletal defects [49,50]. Difficulties in controlling the in vivo release and maintenance of therapeutic activity and the high cost of these growth factors make such strategies less attractive for clinical translation. Alternative strategies that have been investigated include a nanocomposite coating of graphene oxide−copper which was applied to scaffolds of porous calcium phosphate cement. This composite was able to induce bone repair and vascularization when implanted in the rat calvarial defect model [51]. Oliveira et al. [52] were able to improve bone repair and vascularization in a rat condyle defect by the controlled release of Ca^{2+} through the application of an injectable composite comprising of calcium phosphate glass−ceramic with a (hydroxypropyl)methyl cellulose matrix [52].

The use of vascular endothelial progenitor cells (EPCs) has had a significant effect on the neovascularization at bone defect sites [53,54] and when combined with MSCs has significantly improved the osteogenic potential of regenerating bone [55]. Pericytes (which play a role in angiogenesis) and perivascular stromal cells have been implicated as a source of cells capable of bone regeneration [56]. Indeed, Tawonsawatruk et al. [57] showed pericytes derived from adipose tissue improved bone formation when implanted into the site of nonunion bone fracture. The problems associated with the seeding of vascular cells on synthetic composite bone biomaterials are the lack of anastomosis, leakage of primitively formed blood vessels, and cell death due to the slow angiogenic integration of the scaffold. The use of decellularized matrices has tried to circumvent these issues for improving vascularized skeletal repair [58] and was able to demonstrate vascularized bone regeneration by the combination of scaffolds derived from decellularized tendons seeded with human adipose derived stem cells (hADSC)-derived osteogenic cells and human cord blood-derived EPCs [58].

The periosteum is an overlooked but critical part of bone functionality. It has been demonstrated that engineered periosteal tissue can significantly improve bone fracture repair [59,60]. Moreover in an organotypic culture model, placental-derived decellularized blood vessels were employed as a pseudo periosteal sheath improving the repair of bone defects [61]. Lack of specific porosity and tight integration of tissue-engineered scaffolds at the defect site, or in the case of hydrogels, the leakage of gels into the surrounding tissue has been a problem for vascular bone repair strategies. With the development of better technologies such as 3D bioprinting and material design, production has significantly improved to create tailored complex vascular material structures for the engineering of bone tissue [62]. Cui et al. [63] demonstrated an example of this, whereby complex structures can be printed with functionalized porous scaffolds incorporating VEGF and BMP-2 perfused with human umbilical vein endothelial cells (HUVECS) and MSCs [63]. In addition, to provide better integration and minimize cell death in large bone defect/scaffold implants, the engineering of prevascularized scaffold composites can potentially overcome these quandaries. Bioprinting techniques have been used to create complex porous structures with integrated channels and MSCs [64]. These scaffolds could be actively perfused for longer than 6 weeks with osteogenic growth factors. In addition, Klotz was able to develop a multifaceted composite in which endothelial colony-forming cells and MSCs were seeded to a central channel of 600 μm and hydrogel respectively. The self-assembling capillary networks that developed in the osteogenic construct demonstrated the potential prevascularization of large bone scaffold tissues [65]. The potential of these strategies on a large scale was demonstrated by the use of a sheep arteriovenous-loop model to regenerate bone by combining MSCs, BMP-2, and β-tricalcium phosphate-hydroxyapatite [66].

Ongoing developments in bone regenerative strategies, coupled with a better understanding of the interactions between vasculature and bone processes, are necessary toward achieving the clinical translation of engineered bone for the repair of large bone defects, as well as the screening of new biomolecules for use in bone reparation.

Preclinical development—ex vivo/in vivo small and large animal preclinical models

Progressive bone tissue regenerative technology such as 3D bioprinting and material functionalization requires robust successful testing to understand the potential of these complex composites and the interaction of skeletal regenerative cells before translation into the clinical environment. Autologous bone grafting sets the gold standard

for bone defect repair both in preclinical and clinical models. However, there are limitations to the use of autologous and allogenic bone grafting. Alternative bone regenerative strategies have included the direct application of stem cells or skeletal progenitors into bone defect sites, usually in combination with functionalized osteogenic inducing bioscaffolds such as demineralized bone matrix, polymethylmethacrylate, hydrogels, polymers, and ceramic scaffolds [67,68]. In vitro models inform essential ex vivo and in vivo studies into bone regenerative therapies [69,70]. Whereas preclinical models provide the 3D environment, vascularization and immunology gain insights into the potential outcomes of regenerative strategies in repairing and regenerating bone defects. Indeed, bone pathology that arises from many disease processes, for example, nonhealing fractures, osteoporosis, neoplasia, dental tissues, have been replicated in animal models [71−73].

Successful regenerative outcomes of skeletal defects using stem cell therapies in combination with biomimetic scaffolds or hydrogels rely on the material being biocompatible, bioactive, and biodegradable without adverse tissue or toxicity interactions [74]. Hence, the principles of bone regeneration via osteoconduction, osteoinduction, and osteointegration are key. Examples of these biomaterials in preclinical bone repair models include the use of chondroitin-sulfate glycosaminoglycan scaffolds as a delivery vehicle for BMP-2 [75] and a biomaterial-based scaffold (macroporous gelatin−calcium sulfate−hydroxyapatite) with osteoinductive rhBMP-2 and zoledronate to repair a rat tibial defect [76]. Careful examination of biomimetic scaffolds in vivo should be carried out early in the study material development pathway to avoid wasting time and resources pursuing materials that are not viable. For example, collagen-1-based scaffolds have been demonstrated to negatively impact fracture healing by inhibiting mineralization during osteogenic differentiation [77].

Factors to be considered when selecting the animal type for a bioengineering bone defect repair in vivo study include ease of handling and intensity of care, uniformity of breeds, similar biology to humans, lifespan, and speed of healing. Rodent models have many advantages; however, they differ from human bone by the lack of Haversian systems [78]. Depending on the study objective, preclinical rabbit models are used and sheep have become more popular as the large animal model of choice for clinical translation. Mills & Simpson [79] describes the variance in these models for normal healing fracture repair, delayed union, nonunion, segmental defects, and fractures at risk of impaired healing.

"Implantation models" provide the first indications of the biocompatibility and osteogenic induction of materials and stem cells, an essential step in the pathway to clinical translation. The organotypic (avian, rodent) bone culture ex vivo system and the avian chorioallantoic membrane vascular implantation model provide an initial, rapid assessment of the function and biocompatibility of materials and cell constructs for bone regeneration [80]. Implantation models involve siting scaffolds or ECM-like materials with or without adjunctive agents and/or cells into an ectopic site, for example, subcutaneously or into a muscle pouch [81]. Fig. 50.3 depicts the osteogenic development of various manufactured (casted or 3D−printed) scaffolds [Laponite−alginate−methylcellulose (3−3−3)] by microcomputed tomography scanning over an 8-week period when subcutaneously implanted into immunocompromised mice.

Another implantation model includes a calvarial defect model, a nonloading bone defect model that has been employed successfully to assess the osteogenic capacity of biomaterials for bone regeneration. Improved rates of repair in a critical-sized calvarial defect were achieved by the combined implantation of bone marrow stromal cells (BMSCs) and vascular cells [82]. Furthermore, in a rabbit critical-sized calvarial defect model, repair was achieved by a composite hybrid scaffold composed of MSC sheets, hydroxyapatite, and platelet-rich fibrin granules [83]. Lee et al. [84] described a 3D printed kagome-structure scaffold using a rabbit calvarial defect model which enhanced osteoconductivity and was robust when mechanically tested. In a comparative study the tibia and parietal bones were compared showing greater "neobone" formation in the tibia due to a higher vascular input and mechanical load compared to the parietal defects which derive vascularization and osteogenic cells from the dura mater [85].

In vivo spinal fusion models have been employed to develop clinical treatments primarily to augment spinal fusion to increase the success rate of fusion and decrease the need for secondary interventions. Rat models have been successfully used in posterolateral lumbar spinal fusion studies to assess pharmacological agents, gene therapy, and growth factors including BMPs. Studies have found that 10 μg rhBMP-7 added to collagen bone matrix resulted in a clinically solid fusion as early as 3 weeks postoperatively, compared to 3 μg rhBMP-7, which showed new bone formation but no solid fusion mass [86]. Recently, successful bone formation in a rat posterolateral fusion model was achieved with platelet-rich plasma combined in a collagen-mineral scaffold compared to that of platelet-poor plasma or scaffold only groups [87]. Anterior lumbar spinal fusion was modeled in sheep using an rhBMP-2 collagen composite. The rhBMP-2 group was found to be 20% more stiffer and had three times higher histologic fusion rates compared to autograft [88].

Load bearing bone defects in rodents have been the driving force in ascertaining the potential therapeutic

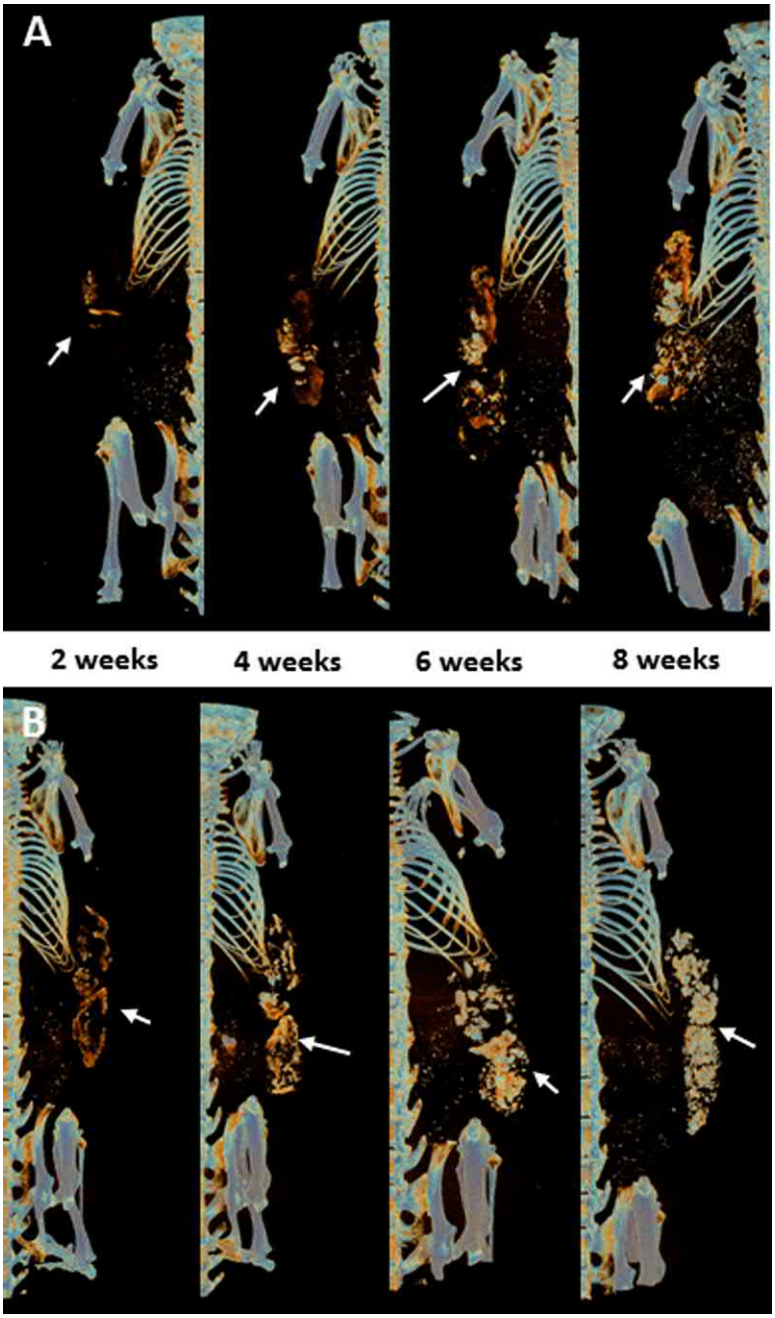

FIGURE 50.3 Sequential micro CT scanned images (2−8 weeks) of subcutaneous implanted scaffolds into an immunocompromised mouse. (A) Casted Laponite−alginate−methylcellulose (3−3−3) + skeletal stem cells; (B) 3D-printed Laponite−alginate−methylcellulose (3−3−3) + skeletal stem cells (*arrows* depict mineralized scaffold). *Courtesy Dr. Gianluca Cidonio, Bone and Joint Research Group, University of Southampton (Unpublished data).*

activity of various composite biomaterials for bone repair. In a unicortical femoral defect model in the rat, significant levels of osteogenesis were achieved by the injection of a hyaluronan hydrogel with encapsulated BMP-2 [89]. In critical-sized bone defects, combinations of vascular cells and skeletal stem cells with functionalized scaffolds have been shown to significantly improve bone repair and vascularization [50,90]. Of relevance, infection of bone defect sites is a critical problem, particularly when implanting artificial prosthesis or biomaterials. Nanosilver−PLGA (poly(lactic-co-glycolic acid) composite BMP-2 scaffolds were found to induce bone formation with significant antibacterial (antimicrobial) properties when implanted into a rat femur defect [91]. However, if

incorporated, antibiotic release from a scaffold should continue until the material is degraded to ensure it does not act as a nidus for infection [92].

Rabbits are often used prior to progressing to larger animals as rabbit bone contains Haversian systems and so replicates large animal models of bone structure. However, they have a higher capacity for increased bone turnover making it difficult to assess healing rates compared to humans [93]. Anand et al. [94] investigated mesoporous bioactive glasses with surfactants in rabbits and found them to be nontoxic, biodegradable, and biocompatible with hexadecyltrimethylammonium bromide and were the most effective material investigated in regenerating new bone. A 3D printed polylactide-*co*-glycolide/tricalcium phosphate composite scaffold incorporating magnesium powder was employed in a rabbit radii segmental defect study and was biocompatible and enhanced bone regeneration [95]. Mini-pig bone (tibial) defects have been successfully regenerated via a two-step therapy by implanting MSCs seeded collagen sponge and injecting microbubbles with BMP-6 plasmid DNA at later time points and activating these molecules by ultrasound [96]. Moreover, microvesicles derived from stem cells containing mediators of paracrine effects can be used to communicate with cells to enhance bone regeneration. Hence, scaffolds with the capability of microvesicle delivery are potential therapies for bone defect repair [97].

For successful clinical translation of bone tissue engineering therapeutic constructs, preclinical models need to replicate the surgical procedure and biomechanical forces that the implanted biomaterials will endure [98]. Sheep are a similar bodyweight to humans with bone dimensions amenable to fixation and biomaterial studies. However, ovine bone slightly differs from human bone with seasonal and age variation [93]. The osteochondral and tibial defect sites in large animals have provided the optimal model to investigate critical sized defect repair [99]. However, in most cases these large models are far from standardized making it difficult to compare differing bone tissue engineering reparative strategies. Autograft, rhBMP-7, and scaffolds (medical-grade polycaprolactone and tricalcium phosphate) with BMSC have been successfully tested in an ovine critical-sized defect model [100]. Moreover, at the site of an iliac crest bone defect, the application of encapsulated MSCs in a composite RGD (Arg-Gly-Asp)-hydrogel of alginate and hyaluronate containing biomineralized polymeric microspheres significantly increased vascularization of the defect site with increased osteoid and bone formation occurring compared to the controls [101]. Many other studies, not included in this section, that have a skeletal pathophysiology are relevant models for investigating regeneration of fractures. For example, there are osteoporotic models whereby MSCs strontium hydroxyapatite engineered scaffolds have induced osteogenesis in a bone defect site [102]. When choosing these models it is worth noting that metaphyseal bone fracture healing differs from diaphyseal bone healing.

In vivo studies are key in translating potential bioengineering treatments to ensure the skeletal regenerative materials are safe, robust, and efficacious for clinical use. It is vital to include positive and negative controls for each experiment such as a novel agent compared to bone graft and a nontreatment control [81]. Pilot studies to determine variation within the model, assess control treatments and allow surgical planning and technique are crucial for in vivo experimental design and statistical power calculations [81]. These steps will help standardize the multitude of bone tissue engineering preclinical models, providing robust comparable data sets between research models and the new bone biomaterials being developed for potential translation to the clinic.

Clinical translation

The current gold standard for stimulation of fracture union or arthrodesis is autograft, while autograft or allograft is commonly used in clinical practice to replace lost bone stock. Autograft is osteoconductive, osteoinductive, and does not provoke an immunogenic reaction, although associated donor site morbidity limits its use. For larger procedures, allograft is the material of choice, but holds significant biological, economical, and practical disadvantages to its application. Thus tissue engineering and regenerative medicine have come to the fore in recent years in an attempt to address the unmet need for skeletal repair and bone augmentation. Tissue engineering approaches were categorized as (1) use of cells, (2) use of tissue inducing substances, and (3) delivery of cells within a matrix [103]. Significant innovation has taken place in the decades since these seminal articles, including the application of nanotopography to modulate in situ cell responses [74]. It is intuitive that different clinical problems such as a fracture nonunion and an osteochondral defect represent distinct biological problems requiring distinct tissue engineering strategies. However, less obvious, but of critical importance is that within a single clinical problem, for example, fracture nonunion, a variety of problems may prevent healing. Thus patient selection is critical to enable successful application of a tissue engineering strategy. For example, atrophic nonunion, exemplified radiographically by poor callus formation represents a failure of bone biology. In contrast, hypertrophic nonunion in which copious callus is observed radiographically represents a failure of mechanical stability. A trophic nonunion would be an appropriate target for a tissue engineering strategy designed to stimulate bone cells, in contrast this would be ineffective in cases of

biologically active hypertropic nonunion. Indeed, the phrase "right patient, right operation, right time" may be adapted in the context of tissue engineering "Right tissue engineering strategy for the right biomechanical problem." In this section, clinical translation of tissue engineering strategies in metabolic bone disease, stimulation of union (post fracture and arthrodesis), bone defects, and osteochondral defects are discussed.

Modulation of in situ cellular activity by denosumab, a monoclonal antibody has been used for the treatment of osteoporosis for many years [104]. More recently an additional monoclonal therapy, burosumab has been approved for the treatment of X-linked hypophosphatemia [105]. Denosumab inhibits resident osteoclasts, while Burosumab increases renal absorption of phosphate via an inhibitory effect on fibroblast growth factor 23. While these treatments are efficacious and novel, they represent isolated manipulation of an in vivo pathway rather than cell selection or application of a cell-laden scaffold. In contrast, BMP, a potent osteoblast stimulator, has been applied within a bovine-derived collagen sponge and used to stimulate fracture healing for many years [106]. However, this treatment has not been widely adopted and subsequent meta-analysis has suggested there is limited evidence to suggest that BMP may be more effective than controls for acute tibial fracture healing. While the evidence for the use of BMP in treating nonunion remains unclear, limited evidence suggests that BMP use may be appropriate in treatment of acute high energy tibial fractures at high risk of nonunion with standard treatment [107]. BMP-2 delivered in a collagen sponge combined within a titanium cage (INFUSE) has been approved since 2002 by the FDA to mediate intervertebral fusion. INFUSE has shown efficacy in randomized controlled trials when compared with iliac crest autograft in mediating spinal fusion. However, a number of significant side effects, including heterotopic ossification, possibly related to the use of supraphysiological doses [108] appear to have precluded more widespread use.

A plethora of synthetic bone graft materials have been produced over the decades to treat both bone loss and stimulate union or arthrodesis. Synthetic bone has been produced using acid-mediated demineralization of bone matrix—resulting in demineralized bone matrix, hydroxyapatite $Ca_5(PO_4)_3OH$, calcium phosphate $Ca_3(PO_4)_2$, calcium sulfate, coral, bioactive glass—and from polymers such as poly(methyl methacrylate) and polylactic acid. In clinical practice, bone graft may be required to fulfill distinct objectives such as regeneration of bone stock, for example, during arthroplasty revision for aseptic loosening; stimulation of healing in nonunion; and provision of mechanical stability in tibial plateau fractures. As such, the optimal characteristics of a bone graft will depend upon the clinical requirements of a particular patient and their fracture or disease. At present, synthetic products lack osteogenicity associated with autograft, and attempts are being made to overcome this problem by combining synthetic graft with autologous BM aspirate.

The pathogenesis of atraumatic osteonecrosis of the femoral head, resulting in bone loss and bone collapse, remains poorly understood and has been associated with a variety of conditions including steroids, alcohol, sickle cell disease, and Caisson disease (diver's disease). Combination of standard therapy (core decompression of the femoral head) with delivery of autologous BM stromal cells failed to show efficacy in a randomized controlled trial [109]. However, it is conceivable that such an approach may show efficacy in a subgroup of patients due to the variation of pathogenesis of osteonecrosis.

Success of arthroplasty in providing pain relief for millions of patients has resulted in ever greater numbers of patients requiring revision arthroplasty procedures. A recent novel approach has been the use of nanotopography on the implant service to modulate the behavior of resident osteoblasts and promote osteointegration of the implant [74], thereby attempting to reduce revision.

Osteochondral defects, commonly seen in the knee and talus, represent a spectrum of disease ranging from localized areas of cartilage loss to more widespread tissue damage. Cartilage loss may result from a known single episode of trauma or from repetitive microtrauma in which joint stability and mechanical alignment may be causative factors. Osteochondral defects can also result from osteochondritis dissecans, the etiology of which remains unknown. Osteochondral defects may result in pain, swelling, and mechanical symptoms such as "catching" or "locking". Treatment of osteochondral defects is extremely challenging as the cartilage is avascular and subject to significant mechanical loading. A current treatment strategy is microfracture or marrow stimulation in which holes are drilled during arthroscopy in the damaged cartilage to induce bleeding from the underlying subchondral bone. However, this procedure if successful results in the generation of fibrous tissue over the defect rather than biomechanically superior native hyaline cartilage. There is a consensus that this approach is not effective in defects larger than 2 cm^2 [110]. In an attempt to improve tissue regeneration, autologous chondrocyte implantation (ACI) was developed. Initially, this was a two-stage process involving isolation of chondrocytes from nonarticular "donor" region, in vitro culture and subsequent implantation. ACI has demonstrated efficacy in the treatment of osteochondral defects, although the long-term effects remain unknown [110]. Requirement for two surgical procedures, harvesting of "donor" cartilage and in vitro culture, represent major limitations of ACI. To overcome these limitations an approach utilizing synthetic hyaluronic acid scaffolds seeded with autologous BM cells

harvested intraoperatively from the iliac crest has been developed (ABICUS). Efficacy of ABICUS compared to microfracture in the treatment of osteochondral defects has been evaluated in a randomized controlled trial the full results of which are eagerly anticipated [111]. Thus while significant progress has been made in translation of bone tissue engineering strategies in the field, major challenges include evaluation of treatments applied to treat injuries classified under broad clinical diagnostic criteria often as a consequence of an absence of the ability to distinguish variations in underlying biological and mechanical pathophysiology and the unresolved need for simple, efficacious strategies to generate, at scale, vascularized constructs.

Summary and future perspectives

Bone regeneration strategies offer new and alternative therapies for orthopedic applications, including nonunion fracture, healing of critical-sized segmental defects, and regeneration of articular cartilage in osteochondral defects. The ready accessibility of skeletal progenitor and stem populations from BM and the ability of these cells to differentiate into bone-forming osteoblasts when implanted in vivo augers well for translational bone tissue engineering. Key challenges remain the need for facile isolation protocols in the absence of specific markers coupled with safe and efficacious delivery systems to ensure skeletal cell phenotype and function. Despite a multitude of biomaterial scaffold products to aid this process, key challenges remain (1) reduced osteogenicity of synthetic graft materials compared to autograft and (2) development of vascularity within the graft material [112]. While synthetic bone graft materials have been produced with porosity optimized for vascular ingrowth and prepared with BM aspirate to optimize osteogenicity, for example, nanOss [113], evaluation of the efficacy of such products is challenging due to the interpatient variation in quality and quantity of BM aspirate and autograft with which the synthetic product is mixed [113]. Interestingly, the use of synthetic engineered bone products in a number of clinical applications, including (1) filling of voids, (2) fracture stabilization, (3) impaction grafting during arthroplasty surgery, and (4) spinal repair, is supported by randomized controlled trials and various proof of concept studies. However, a significant unmet challenge remains the development of cell-loaded, vascularized constructs for application in large segmental bone defects and in cases requiring large scale bone reconstruction. At present the utility of bone tissue engineering strategies is in part limited by our diagnostic capabilities. Advances in imaging and use of biomarkers may permit early identification of fractures which are likely to fail to unite and potentially indicate a particular biological limiting factor. Such information would allow more specific tissue engineering strategies to be deployed, improving safety, efficacy, and affordability in a financially austere environment.

Future developments of tissue engineering will undoubtedly involve lessons from developmental paradigms of musculoskeletal tissue formation, specifically for understanding developmental biology of bone formation, particularly in the adult context of injury and disease.

To provide a step change in the field will necessitate interdisciplinary and multidisciplinary working with the cross fertilization between clinicians and life scientists paramount to aid our understanding of the continuum of skeletal cell development, skeletal niche, skeletal cell plasticity, and skeletal reparation. These are exciting times, bone tissue engineering promises to deliver specifiable replacement tissues, with significant orthopedic application. Although, there remains for many a significant gap in translation to the clinic, the judicious selection of skeletal populations, biomaterial scaffolds, and generation of vascularized skeletal constructs at scale is a goal worth pursuing, not least given the opportunities to improve the quality of life for an aging population demographic.

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Chapter 51

Tissue engineering for regeneration and replacement of the intervertebral disk

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Introduction

The intervertebral disk (IVD) is the fibrocartilaginous part of a "three-joint complex" that governs motion, flexibility, and weight-bearing in the spine (Fig. 51.1). As a part of this complex the disk undergoes a lifetime of "wear and tear" that contributes to multiple IVD disorders of enormous consequence for human disability and suffering. These IVD disorders are poorly understood musculoskeletal pathologies characterized by multiple anatomic features, including internal disk disruption, loss of IVD height, IVD tears, IVD dehydration, and the generation of herniated disk fragments [1–3]. These anatomic features may associate with nerve root compression or irritation, spinal canal narrowing (stenosis or spondylolisthesis), or facet joint impingement that contribute to symptoms of low back pain, neurological deficits, and disability that affect between 4% and 33% of the US population annually and have a mean global lifetime prevalence of 38.9% [4–6]. Like most cartilaginous tissues, the IVD is an avascular and a lymphatic structure that exhibits little to no capacity for repair following injury and experiences aging-related cell density losses that may further limit biologically mediated repair [7]. The extreme mechanical demands on the IVD also contribute to tissue failure and degeneration, due to the high magnitudes of compressive, tensile, and shear stresses and strains that result from joint loading, muscle activation, and spinal flexibility. As a result, strategies to intervene in the progression of IVD disorders are met with significant biological and mechanical challenges that frustrate success.

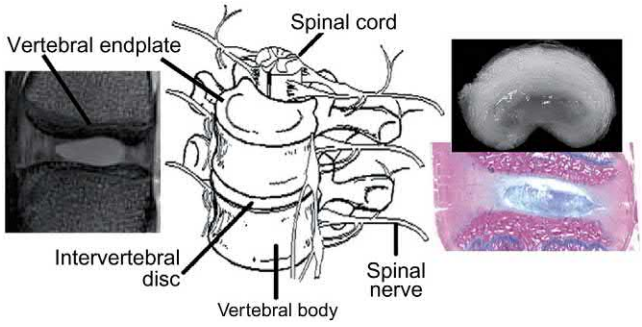

FIGURE 51.1 Schema of spinal motion segment illustrating location of intervertebral disk between superior and inferior vertebral bodies. Image at left MRI appearance of immature lumbar disk with characteristic intense nucleus pulposus region. Images at right illustrate (top) gross appearance of nondegenerate lumbar disk and (bottom) histological appearance of immature disk in a stained section. *MRI*, Magnetic resonance imaging. *Modified schema reprinted with permission from Columbia-Presbyterian Neurosurgery.*

Numerous surgical procedures have been developed to treat IVD disorders, which rely almost completely upon reducing motions across the disk space to restore stability during weight-bearing. A large number of devices have been developed that promote bony fusion processes across the disk space, with less attention being given to mobile-bearing artificial disk replacements and dynamic stabilization devices that allow but limit motions [8]. In cases where the pathology permits, removal of extruded IVD fragments may be performed in a procedure termed as discectomy. Together, these procedures comprise more than 300,000 inpatient hospitalizations annually in the United States alone [9]. The clinical practitioner would not typically entertain the idea of fusing or plating most pathological joints in the body, such as the hip, knee, or shoulder, although this is our 21st century standard of

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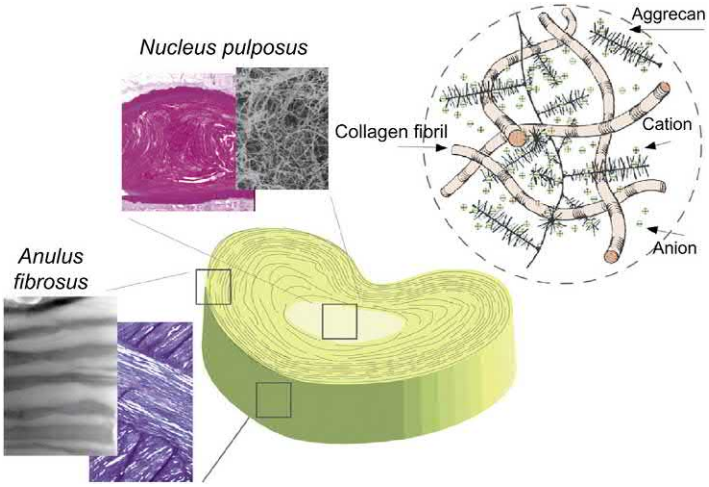

FIGURE 51.2 Schema of different zones and microstructures comprising the intervertebral disk.
Annulus fibrosus insets of macroscopic appearance and stained section illustrate the lamellar structure of the tissue. The lamella comprises aligned collagen fiber bundles that are oriented with alternating angles of ±60 degrees. Nucleus pulposus insets of a stained section and scanning electron micrograph illustrate the randomly organized network of fine collagen fibers and gelatinous nature of the tissue. Circular inset contains a schema of building blocks for these cartilaginous tissues that include banded type I and type II collagen fibrils, aggrecan and smaller proteoglycans, water and multiple ionic species.

care for the pathological IVD. There exists an obvious need to develop alternative strategies to not only treat the consequences of IVD disorders but also to detect and limit the progression of symptomatic IVD pathology.

Success with cellular therapies for articular cartilage regeneration, gene therapy, and in vitro regeneration of cartilaginous tissue has raised hope for tissue-engineered treatments for IVD disorders. Tissue-engineered approaches to IVD regeneration have been focused around implantation of cell-supplemented or acellular biomaterials that may partially or fully replace the IVD structure, as well as delivery of cells or bioactive factors designed to promote the natural repair process. In this chapter a review of these tissue-engineering strategies will be provided along with evaluations of their adaptation and implementation for treatment of IVD disorders.

Intervertebral disk structure and function

In all structures of the IVD the extracellular matrix (ECM) provides physical and biochemical cues that regulate cell-mediated repair or breakdown in mature or aging tissues [10]. The native matrix organization and interaction with the local IVD cell population will be important considerations in the design of any tissue-engineered regeneration strategy. The IVD comprises a centrally situated and gelatinous tissue, the nucleus pulposus (NP), that differs substantially from the more fibrocartilaginous annulus fibrosus (AF), on the radial periphery (Fig. 51.2). On both superior and inferior faces are cartilaginous endplates that provide an intimate mechanical and biophysical connection between the vascularized vertebral bone and the avascular IVD. Both the AF, with a vascularized periphery, and the cartilaginous endplates are believed to be important routes of nutrient transport to all cells of the IVD [11,12]. Given the low cell density of the IVD, maintenance of both cellularity and a generous nutrient supply is often held to be critical to a successful biologically based regenerative strategy.

The immature NP is highly hydrated (>80% water) with ECM components that include randomly organized type II collagen fibers and multiple forms of negatively charged proteoglycans (Table 51.1 [13]). A population of large and highly vacuolated cells is present in the NP during development and growth, with a shift toward a more chondrocyte-like cell population by age 7 [14,15]. Like all IVD regions, the NP contains multiple collagenous and noncollagenous proteins, including types III, V, VI, and IX collagens; elastin, fibronectin; and laminin [16–18]. The NP is largely loaded in compression (Fig. 51.3) and experiences high interstitial swelling and fluid pressures, which arise from joint loading and a high density of osmotically active, proteoglycan-associated negative charges [19,20]. Nachemson showed, as early as the 1960s, that this interstitial fluid pressure is greater than 0.5 MPa (or ~5 times atmospheric pressure) in the NP region [21]. An early loss of hydration or tearing in the NP [3], often detected as a loss of MR signal [22], is believed to contribute to a loss of fluid pressurization in the IVD that may lead to herniation or stenosis with aging [23–25]. With the loss of fluid pressurization the load distribution to the AF will shift from a characteristic outward "bulging" of the annulus to one of inward displacement [26–28]. Partial or complete removal of the NP, occurring in some discectomy procedures, may lead to a loss of disk pressurization and disk height that will transfer loads to facet joints of the spine, increase segmental range of motion, and impact overall spinal stability. Restoration of this interstitial swelling pressure in the NP, or restoration of MR signal intensity, is an oft-cited criterion for the restoration of a healthy functioning disk.

The AF is a lamellar, fibrocartilaginous structure that is highly organized into distinct lamellae of highly oriented and largely type I collagen–containing fiber

TABLE 51.1 **Intervertebral disk (IVD) composition and mechanical properties.**

	Water (wt.%)	Collagen (% dry wt.)	Proteoglycan (% dry wt.)	Other proteins (% dry wt.)	Compressive modulus (MPa)	Shear modulus (MPa)	Tensile modulus (MPa)	Interstitial pressure (MPa)[a]
Nucleus pulposus	70–90	15–35	25–60	20–45	0.5–1.5	0.005–0.01	NA	0.5–3.0
Annulus fibrosus	65–80	10–65	10–35	15–40	0.5–1.5	0.08–0.40	20–50- $\parallel$ circ 0.5–5.0- $\perp$ circ	0.1–1.0
Notes		Types I, II, VI, IX, XI	Aggrecan, decorin, biglycan, fibromodulin, versican, and more					

Ranges reported for compositional features and mechanical properties for nucleus pulposus and annulus fibrosus tissue regions of the nondegenerate IVD. Both composition and mechanical properties of the disk vary substantially with region and with degeneration. Additional mechanical features important to tissue function, such as failure strength, are not shown here.
[a]*Reported also as peak hydrostatic pressures, or swelling pressures.*

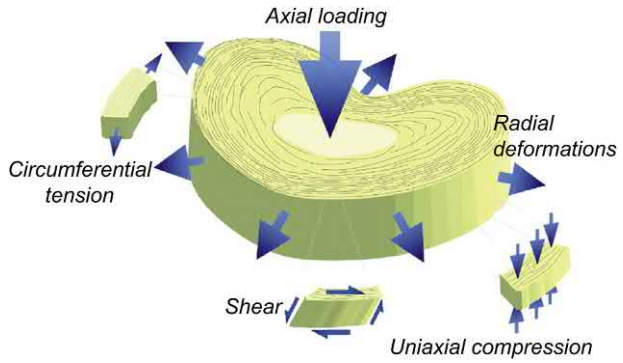

FIGURE 51.3 Axial compressive loading of the intervertebral disk gives rise to a radial deformation, or "outward bulge", as the disk deforms in response to the compressive load.
The high tensile stiffness of the healthy annulus fibrosus in the circumferential direction acts to restrict this outwardly directed deformation. Tissues of the disk will be variably loaded and experience a combination of compression, tension and shear, as shown. Pressurization of the central and gelatinous nucleus pulposus is an important mechanism for load support and load transfer to the annulus fibrosus and contributes to maintenance of disk height.

bundles [29,30]. Type II collagen concentration increases toward the innermost region of the AF, as the concentration of type I collagen is diminished. As with the NP, the AF contains proteoglycans within the collagenous ECM, although at lesser concentrations that vary from outer to inner regions of the tissue. The collagen reinforcement within the AF resists the tensile loads, which arise during physiological joint motions, and the swelling effects, which give rise to significant annular bulging and deformation. Consequently, the AF has a very high stiffness in tension, with moduli that vary with the angle of orientation along the principal collagen fiber direction (Table 56.1 [31–36]). Cells of the AF originate from the mesenchyme and exhibit many characteristics of fibroblasts and chondrocytes [10,37–39]. These cells are sparsely distributed in the mature IVD and exhibit very little intrinsic ability for self-repair. Disorders of the IVD that involve displacement or herniation of an IVD fragment are believed to arise from tears in the AF region, and discectomy procedures frequently involve removing a portion of this annulus tissue. Some tissue-engineering strategies are being developed around the restoration of healthy AF function or composition in part motivated by a need to repair damage subsequent to intradiscal cell or biologics delivery.

The hyaline cartilage endplates of the IVD are important structures that transmit and distribute loads of the spinal column to the disks. Because of their direct contact with both the AF and the NP, the endplates are believed to be an important route of nutrient transport, particularly to cells of the NP [12,40–42]. With aging the cartilage endplate will thin, as it undergoes mineralization and eventual replacement by bone. This mineralization of the endplate is thought to impede diffusion and nutrient flow to the disk, principally the NP that is lacking in an alternate short diffusion pathway. Endplate changes, such as sclerosis, fracture, or modified vascularity, may be detected by magnetic resonance imaging (MRI) changes [43] and are believed to contribute to symptomatic IVD degeneration [44,45]. Thus tissue-engineering strategies that preserve the health of the endplate without inducing additional damage are believed to be critical to restoring IVD function.

Cell-biomaterial constructs for intervertebral disk regeneration

A persistent limitation of acellular, synthetic replacements of the IVD is their biologically mediated or mechanically induced failure due to the harsh loading conditions and bioactivities of cells within the disk space. These challenges are rooted in the fact that the materials used for such applications have no capacity for self-renewal nor self-repair. This has led to increasing interest in tissue-engineering methods to regenerate new IVD in situ or to transplant IVD tissue that has been generated ex vivo. Such strategies have been employed to augment repair of other types of cartilage, most notably articular cartilage and meniscus, which share some features of the harsh biologic and mechanical loading environment within the IVD.

Nucleus pulposus cell-biomaterial implants

As in other cartilage tissue-engineering applications, a main strategy for IVD regeneration has been the inclusion of cells with biomaterials to enable production and long-term maintenance of newly generated tissue. Biomaterials that enable appropriate cellular phenotypes and matrix biosynthesis, and that sometimes enable polymeric degradation or resorption, have been proposed as alternative implantable biomaterials and have been studied largely in vitro. The goals for use of these scaffolds are similar to that for other biomaterial implants, with the additional goals to support native and exogenous cell survival, promote ECM synthesis and remodeling, reduce pathogenic fibrosis, reduce inflammation, and improve spinal stability [46]. Studies of cell-biomaterial constructs cultured in vitro have demonstrated potential for many materials (Table 51.2), including thermosensitive gels such as chitosan, modified chitosans, and elastin-like polypeptides (ELPs) [47–50]; self-associating gels composed of agarose, collagen, and fibrin [51–53]; or modified forms of these same materials, native tissue constructs such as intestinal submucosa [54], crosslinkable alginates, polyethylene glycol, poly(glycolic acids) (PGAs), and more

TABLE 51.2 Representative intervertebral disk (IVD) tissue-engineering studies.

Material	Target tissue	Cell type	Cell density	In vitro/ in vivo	Assessment
PCL[a]	AF and NP	Bovine AF and NP	$5 \times 10^3/cm^2$	In vitro	Histology, SEM, gene expression
Alginate[b]	AF and NP	Porcine AF and NP	$4 \times 10^6/mL$	In vitro	DNA, ECM analysis
Gelatin/C6S/HA[c,d]	NP	Human NP	$20 \times 10^6/mL$	In vitro	Histology, DNA, ECM analysis, gene expression
CPP[e,f]	NP	Bovine NP	$16 \times 10^6/cm^2$	In vitro	Histology, mechanical analysis
Agarose, collagen[g]	AF	Human AF	$0.2 \times 10^6/mL$	In vitro	Histology, ECM analysis
Gelatin, PLA[h]	NP	Porcine NP	$5 \times 10^6/mL$	In vitro	Histology, ECM analysis, gene expression
Collagen/GAG[i,j]	AF	Canine AF	$40 \times 10^6/mL$	In vitro	Histology, ECM analysis
Collagen/HA[k]	AF and NP	Bovine AF and NP	$13 \times 10^6/mL$	In vitro	Histology DNA, ECM analysis, gene expression
Alginate[l,m]	AF and NP	Porcine AF and NP	$1-10 \times 10^6/mL$	In vitro	Histology, gene expression, mechanical analysis
Collagen[n]	AF	Lapine AF	$10 \times 10^6/mL$	In vivo	Histology
Electrospun PLLA (AF), HA (NP)[o]	AF and NP	Human MSC	$20 \times 10^6/mL$	In vitro	Histology, SEM, gene expression, ECM analysis
Electrospun PCL (AF), agarose (NP)[p]	AF and NP	Bovine MSC	$25 \times 10^6/mL$ (NP), 3×10^6/lamella	In vitro	Histology, SEM, DNA, ECM analysis, mechanical analysis
Contracted collagen gel (AF), alginate (NP)[q,r,s]	AF and NP	Ovine AF and NP, Canine AF and NP	$25 \times 10^6/mL$ (NP), $1 \times 10^6/mL$ (AF)	In vivo	Histology, DNA, ECM analysis, mechanical analysis, MRI
PLGA[t]	NP	Canine NP	$10 \times 10^6/mL$	In vivo	Histology, mechanical analysis, MRI, X-ray
Collagen II/hyaluronan/C6S[u]	NP	Rabbit NP	$10 \times 10^6/mL$	In vivo	Histology, MRI
Nanofibrous PLA[v]	NP	Rat NP	$3 \times 10^7/mL$	In vivo	Histology, DNA, ECM analysis, SEM, X-ray
NP cell–derived matrix[w]	NP	Human MSC	$1 \times 10^6/mL$	In vivo	Histology, MRI, X-ray
Pentosan polysulfate[x,y]	AF	Ovine MSC	$10 \times 10^6/mL$	In vivo	Histology, ECM analysis, MRI, X-ray
Collagen II cross-linked with genipin[z]	NP	Rat MSC	$2 \times 10^6/mL$	In vivo	Histology, ECM analysis, SEM, MRI, X-ray
Collagen II cross-linked with riboflavin[aa,ab]	AF	Ovine MSC and AF	$1 \times 10^6/mL$	In vivo	Histology, MRI, X-ray
Alginate/nanofibrous PCL/ porous PCL[ac]	AF, NP, and endplates	Bovine NP and AF, caprine MSC	$20 \times 10^6/mL$	In vivo	Histology, ECM analysis, MRI, mechanical analysis

Representative overview of studies involving cell-scaffold based tissue engineering of IVD using cells obtained from native tissues only. *AF*, Annulus fibrosus; *C6S*, chondroitin-6-sulfate; *CPP*, calcium polyphosphate; *DNA*, deoxyribonucleic acid; *ECM*, extracellular matrix; *GAG*, glycosaminoglycan; *HA*, hyaluronan; *MRI*, magnetic resonance imaging; *MSC*, mesenchymal stem cell; *NP*, nucleus pulposus; *PCL*, polycaprolactone; *PLA*, polylactic acid; *PLGA*, polylactic-glycolic acid; *PLLA*, poly-L-lactic acid; *SEM*, scanning electron microscopy.

[a] *Johnson et al., Eur Spine J, 2006.*
[b] *Akeda et al., Spine, 2006.*
[c] *Yang et al., Artif Organs, 2005.*
[d] *Yang et al., J Biomed Mat Res B, 2005.*
[e] *Hamilton et al., Biomaterials, 2005.*
[f] *Seguin et al., Spine, 2004.*
[g] *Gruber et al., Biomaterials, 2006.*
[h] *Brown et al., J Biomed Mat Res A, 2005.*
[i] *Saad and Spector, J Biomed Mat Res A, 2004.*
[j] *Rong et al., Tissue Eng, 2002.*
[k] *Alini et al., Spine, 2003.*
[l] *Baer et al., J Orthop Res, 2001.*
[m] *Wang et al., Spine, 2001.*
[n] *Sato et al., Spine, 2003.*
[o] *Nesti et al., Tissue Eng, A, 2008.*
[p] *Nerurkar et al., Spine, 2010.*
[q] *Bowles et al., PNAS, 2011.*
[r] *Bowles et al., NMR Biomed, 2012.*
[s] *Moriguchi et al., PLoS One, 2017.*
[t] *Ruan et al., Tissue Eng, A, 2010.*
[u] *Huang et al., Spine, 2011.*
[v] *Feng et al., Tissue Eng, A, 2012.*
[w] *Yuan et al., Biomaterials, 2013.*
[x] *Oehme et al., J Neurosurg Spine, 2016.*
[y] *Daly et al., Spine J, 2018.*
[z] *Zhou et al., Acta Biomat, 2018.*
[aa] *Hussain et al., Neurosurgery 2018.*
[ab] *Moriguchi et al., Acta Biomat, 2018.*
[ac] *Gullbrand et al., Sci Trans Med, 2018.*

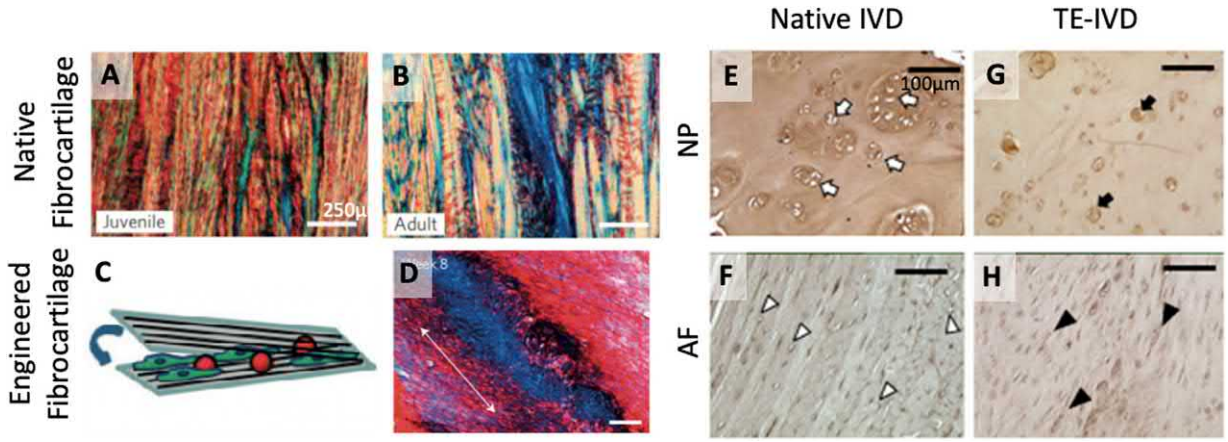

FIGURE 51.4 (A and B) Picrosirius red/alcian blue histology of fibrocartilage from juvenile and adult bovine menisci showing the development of proteoglycan-rich microdomains between bundles of collagen fibers. (C and D) A tissue-engineered strategy employing fibrochondrocytes and mesenchymal stem cells that recapitulates the heterogeneous proteoglycan distribution of fibrocartilage. (E–H) Safranin-O histology 16-week postoperation after implantation of a TE IVD and the adjacent native IVD. The intact IVD shows rounded chondrocyte-like cells in the NP and elongated fibroblast-like cells in the AF, which is largely mimicked by the TE IVD after a 16-week period in vivo. *AF*, Annulus fibrosus; *IVD*, intervertebral disk; *NP*, nucleus pulposus; *TE*, tissue-engineered. *Adapted from (A–D) Han WM, Heo SJ, Driscoll TP, Delucca JF, McLeod CM, Smith LJ, et al. Microstructural heterogeneity directs micromechanics and mechanobiology in native and engineered fibrocartilage. Nat Mater 2016;15(4):477; (E–H) Moriguchi Y, Mojica-Santiago J, Grunert P, Pennicooke B, Berlin C, Khair T, et al. Total disc replacement using tissue-engineered intervertebral discs in the canine cervical spine. PLoS One 2017;12(10):e0185716.*

[46,55–59]. More recently, decellularized NP tissue and NP cell–derived matrices have been investigated due to their mimicry of the native tissue [60–62]. In vitro studies with the above materials are based on evaluating new matrix formation, cell proliferation, differentiation potential, and sometimes degradation characteristics. Hydrogels, such as alginate and gelatin, have been used most commonly for engineering NP tissue, likely due to the fact that such materials reasonably approximate the gel-like properties of the native tissue. Cells of different origins, including native IVD cells, stem cells, and chondrocytes, are capable of synthesizing and depositing collagen and glycosaminoglycans (GAGs) within these hydrogels, although there is little agreement upon the targeted composition necessary to achieve a satisfactory tissue construct. This is a particularly challenging determination for the IVD as the matrix contains varying amounts of both types I and II collagen, so that the exclusive presence of type II collagen does not serve as a phenotypic matrix marker as is the case for articular cartilage.

Annulus fibrosus repair and regeneration

Sealing AF defects is critical after discectomy for disk herniation or implantation of NP replacements, as annular injury has been shown to lead to further IVD degeneration, and integrity of the AF is necessary to retain implanted NP materials [63,64]. While mechanical devices and suturing techniques to seal AF defects are effective for preventing herniation, they do not promote tissue healing and ultimately cannot prevent IVD degeneration [65,66]. Efforts to regenerate the AF have focused on materials, cells, and biomolecules that are able to both restore integrity to the AF to prevent herniation and promote long-term healing of the tissue. Natural and synthetic biomaterials have been investigated for AF repair and regeneration, including hydrogels composed of alginate, agarose, gelatin, hyaluronic acid, and collagen [64,67–70], as well as materials that mimic the native fiber and porous architecture of the AF, including decellularized AF, PGA, polylactic acid (PLA), poly(ε-caprolactone), poly(vinylidene difluoride), collagen, silk, hyaluronic acid, and/or GAGs [71–77]. Recent investigations have documented that this fibrocartilage is heterogeneous, with a distinct microstructure containing proteoglycan-rich microdomains dispersed between fibrous, collagen-rich tissue [78]. Han et al. developed a tissue-engineered construct that mimicked the heterogeneity of fibrocartilage, which appears to be critical to achieving similar mechanical and mechanobiological properties of native tissue (Fig. 51.4). Oftentimes, the same scaffolds evaluated for NP cells are also studied with cells of the AF, with findings that generally illustrate the importance of cell origin in determining the resultant ECM synthesis. A common observation, however, is that cells of either origin that are maintained in a rounded morphology tend to generate more type II collagen, characteristic of hyaline cartilage, whereas those that are cultured in an elongated morphology generate more type I collagen. Moriguchi et al. implanted a collagen/alginate whole IVD in the canine cervical spine and found that

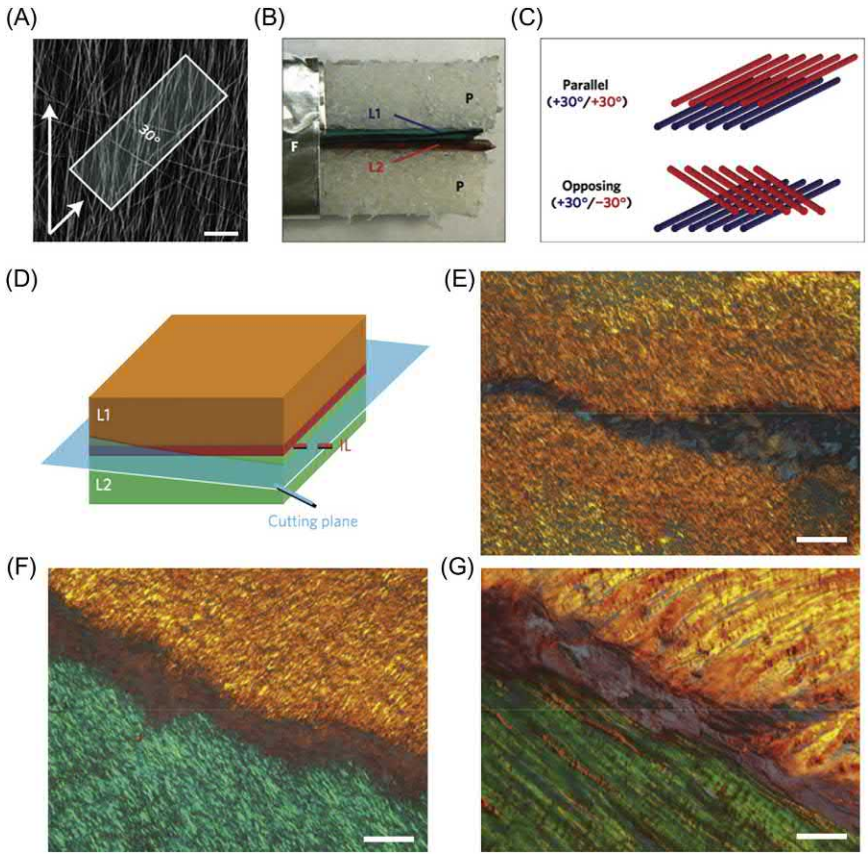

FIGURE 51.5 Fabrication of fiber oriented AF tissue constructs.
(A) Scaffolds were excised 30 degrees from the prevailing fiber direction of electrospun nanofibrous mats to replicate the oblique collagen orientation within a single lamella of the annulus fibrosus. Scale bar: 25 μm. (B) At 0 week, MSC-seeded scaffolds were formed into bilayers between pieces of porous polypropylene and wrapped with a foil sleeve. *F*, Foil; *L1/2*, lamella 1/2; *P*, porous polypropylene. (C) Bilayers were oriented with either parallel (+30/ + 30 degrees) or opposing (+30/ − 30 degrees) fiber alignment relative to the long axis of the scaffold. (D) Sections were collected obliquely across lamellae, stained with Picrosirius Red, and viewed under polarized light microscopy to visualize collagen organization. When viewed under crossed polarizers, birefringent intensity indicates the degree of alignment of the specimen, and the hue of birefringence indicates the direction of alignment. *IL*, Interlamellar space; *L1/2*, lamella 1/2. (E) After 10 weeks of in vitro culture, parallel bilayers contained coaligned intralamellar collagen within each lamella. (F and G) Opposing bilayers contained intralamellar collagen aligned along two opposing directions (F), successfully replicating the gross fiber orientation of native bovine annulus fibrosus. (G) In engineered bilayers, as well as the native annulus fibrosus, a thin layer of disorganized (nonbirefringent) collagen was observed at the lamellar interface. The distribution of collagen fiber orientations was determined by quantitative polarized light analysis [45]. Scale bars: 200 μm (B and C), 100 μm (D). *AF*, Annulus fibrosus; *MSC*, mesenchymal stem cell. *Figure modified with permission from Nerurkar et al. Nat Mater, 2009.*

cells in the collagen region were elongated like fibrochondrocytes found in the AF, while cells in the alginate region were rounded and appeared similar to NP chondrocyte-like cells [79] (Fig. 51.4).

A main challenge has been reproducing the intricate lamellar arrangement of collagen fibers that give the AF its unique mechanical properties and the cells their unique morphology. For this reason, AF tissue-engineered materials have moved toward scaffolds that mimic the apparent collagen architecture. Some investigators have developed synthetic and natural polymeric scaffolds with anisotropic features such as an oriented honeycomb structure [80], aligned collagen gel fibers [81,82], oriented electrospun fibers [83−86], and oriented silk fibers [87]. One approach is based on engineering AF to contain oriented and lamellar electrospun polycaprolactone (PCL) fibers with tensile mechanical properties on the order of native AF [71] (Fig. 51.5). These results are indeed suggestive of the potential to engineer anisotropic collagenous tissues.

While tissue-engineered AF repair strategies have not reached the clinic, there are numerous preclinical in vivo studies in both small and large animal models to repair AF defects. A large percentage of these studies have taken place in the sheep lumbar spine, where tissue-engineered repair strategies are applied after mechanical injury to the AF that simulates human IVD herniation and degeneration [64,88−91]. One such study demonstrated the usefulness of using a tissue-based strategy to repair an AF defect by patching AF defects with small intestinal

submucosa (SIS) anchored by titanium bone screws [92]. The SIS patch produced new integrated tissue at the defect site and increased the capacity of the IVD to pressurize post anulotomy and increased disk hydration compared to anulotomy-only levels after 24 weeks in an ovine model. Other studies have demonstrated closure of AF defects and prevention of degeneration after discectomy in rat tail and sheep lumbar spine models using cross-linked high-density collagen [68,69,88,93,94]. This method involves injection of a collagen hydrogel into AF defects, followed by photo-crosslinking to form the hydrogel to the shape of the defect and increase the strength of the seal. In another study, genipin cross-linked fibrin hydrogels were developed that demonstrated mechanical properties on the order of AF, maintained cell viability, and maintained adhesion to native AF tissue under physiological load [95–98]. While these studies and others with different materials have shown excellent repair of the outer AF, it remains a challenge to effectively repair the inner AF. A potential solution may be the combination of cells, biomolecules, and biomaterials to orchestrate more effective healing of the entire AF [88,91,99,100]. Biomolecules such as bone morphogenetic protein (BMP) 13 have been shown to locally regenerate tissue when applied to AF defects [101]. Further in vivo characterization of these strategies is necessary prior to implementation in the clinic.

Composite cell-biomaterial intervertebral disk implants

Tissue-engineered strategies targeting the NP and AF are primarily aimed toward early-stage IVD degeneration, with the goal of halting degeneration and regenerating the native tissue. Composite tissue-engineered IVD strategies are typically seen as treatments for end-stage degeneration and an alternative to spinal fusion, where there is little salvageable tissue and an entirely new IVD is necessary. Similar synthetic and natural materials are used for the NP and AF regions as discussed previously, often seeded with primary NP and AF cells. Tissue-engineering whole composite IVDs are a complicated endeavor due to the necessity to recapitulate both the gelatinous nature of the NP and the circumferentially fibrous architecture of the AF. In addition, the composite construct must integrate with the surrounding tissue and mimic the mechanical response of native IVDs under varied and complex loads. As such, in vivo testing is necessary to fully characterize how the composite structure integrates with native tissue.

Recent studies have explored the development of composite IVDs using a variety of materials and techniques. Composite IVDs have been produced with AF composed of PGA/PLA, electrospun poly-L-lactic acid, contracted collagen gel, silk, electrospun PCL, and demineralized bone matrix gelatin and NP composed of alginate, hyaluronic acid, hyaluronic acid/fibrin, agarose, silk hydrogel, and collagen II/hyaluronate/chondroitin-6-sulfate [82,87,102–106]. In one of the earliest examples of an integrative tissue-engineering approach, investigators generated NP tissue in vitro by culturing primary bovine NP cells at high density upon a calcium polyphosphate substrate, in order to mimic the natural integration of the NP against the vertebral endplate (Fig. 51.6 [107]). The NP cells formed tissue with a proteoglycan, but not collagen content matched to that of the native NP. Importantly, functional properties in some testing configurations approached that of the native tissue. Gullbrand et al. recently demonstrated effective IVD replacement in the rat caudal spine and goat cervical spine in vivo using agarose/nanofibrous PCL/porous PCL composites [108]. These constructs mimicked the NP, AF, and cartilage endplates and were shown to mature and match native IVD mechanical properties over a period of 20 weeks. While the goat model required external fixation, this is a significant translational step toward bringing tissue-engineered total IVD replacement to the clinic. Additional work will be required in adapting composite tissue-engineering approaches to ensure that mechanical integration with adjacent tissues is adequate.

In studies conducted by Bonassar et al., IVD regeneration was attempted in the native disk space of an athymic rat with a fully integrated scaffold combining contracted collagen gels as a scaffold for AF, and a cross-linked alginate hydrogel as a scaffold for NP tissue [81,82] (Fig. 51.7). Primary cells for culture within each scaffold region were derived from the corresponding native IVD tissues, and the resultant cell-laden scaffolds were implanted in place of the native disk in athymic rats for a period of 6 months. The results illustrate spatially directed matrix regeneration with ECM that exhibited distinct morphologies, contained native levels of collagen and GAGs, and was functionally integrated with native tissue. In biomechanical tests the motion segments containing the composite tissue-engineered disks were found to have a compressive modulus and permeability to flow similar to the native motion segments. Thus this approach illustrated an ability for cell-laden scaffolds to regenerate integrated functional ECM with some of the functional and compositional features of the native tissue in the native disk space. More recent studies in the beagle cervical spine showed similarly promising results; however, this study demonstrated the importance of animal model anatomy [79]. Extreme curvature at certain levels in the beagle cervical spine led to the displacement of the implanted tissue-engineered IVDs, while no displacement and excellent integration were observed in levels without excessive curvature.

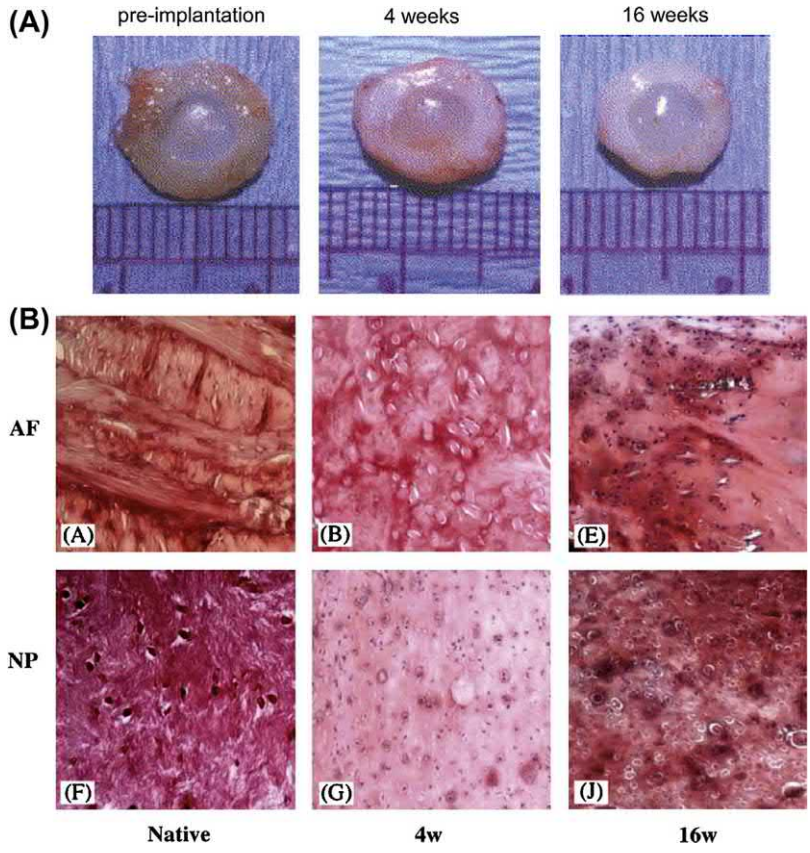

FIGURE 51.6 Histological appearance of in vitro–formed tissues showing newly generated NP tissue. Histological appearance of (A) in vitro–formed cartilage at 2 weeks (time at which the NP cells would be seeded); (B) in vitro–formed NP–cartilage–calcium pyrophosphate composite (triphasic construct) at 8 weeks following seeding of chondrocytes; and (C) in vitro–formed cartilage tissue alone (biphasic construct) at 8 weeks. Arrowheads indicate tissue growing within the pores of the CPP; arrow indicates interface between cartilage and NP tissue (toluidine blue stain; original magnification ×50). *CPP*, Calcium polyphosphate; *NP*, nucleus pulposus. *From Hamilton DJ, et al. Biomaterials 2006;V27:397–405.*

Assessment of the success of IVD tissue-engineering efforts is critical to moving these technologies toward clinical application. To date, the most common tool for the assessment of newly generated IVD tissue has been histology, although analysis of mechanical properties, gene expression, and ECM composition has been frequently employed to confirm the appropriate phenotypic behavior in engineered IVD. In vitro studies have laid the foundation for necessary and/or sufficient characteristics of a successful scaffold for AF and NP replacement. From these studies, for example, it is evident that a high starting cell density and a high degree of initial matrix stability are essential for promoting long-term construct stability and matrix accumulation to eventually restore mechanical function and swelling pressure [109]. Mechanical analysis of engineered IVD tissue is essential in assessing the formation of functional IVD tissue, however both for NP implants—that must restore spinal stability—and annular repair strategies—that must withstand repeated cycles of nucleus pressure. In vivo studies may also evaluate an ability to restore disk height as a common outcome variable, as well as clinical MRI imaging to access in vivo tissue hydration. In vivo, largely preclinical work has begun to verify that engineered IVD tissues may eventually be capable of restoring disk height, maintaining spinal stability and flexibility and retarding disk degeneration in pathological tissues.

Cellular engineering for intervertebral disk regeneration

Given the relatively small numbers of studies in the area of IVD tissue engineering, there is a surprising amount of breadth to not only the biomaterials but also the cell sources utilized for regeneration. The question of cell source is of particular note for IVD tissue engineering, given that the availability of autologous disk cells is extremely low in the adult and that the phenotype of cells varies so substantially with both spatial position and with age. In animals studied for IVD tissue engineering ex vivo the origin of cells in the NP may be partly notochordal or mesenchymal, depending on the age of the animal in question. As such, the choice of species used as a source of cells may be quite important. Due to the ease of availability, porcine and bovine cells are the most commonly used, with other efforts reporting the use of cells of canine, lapine, and ovine origin as well as human. However, cells derived from bovine or ovine tissues may be exclusively mesenchymal in origin, while those

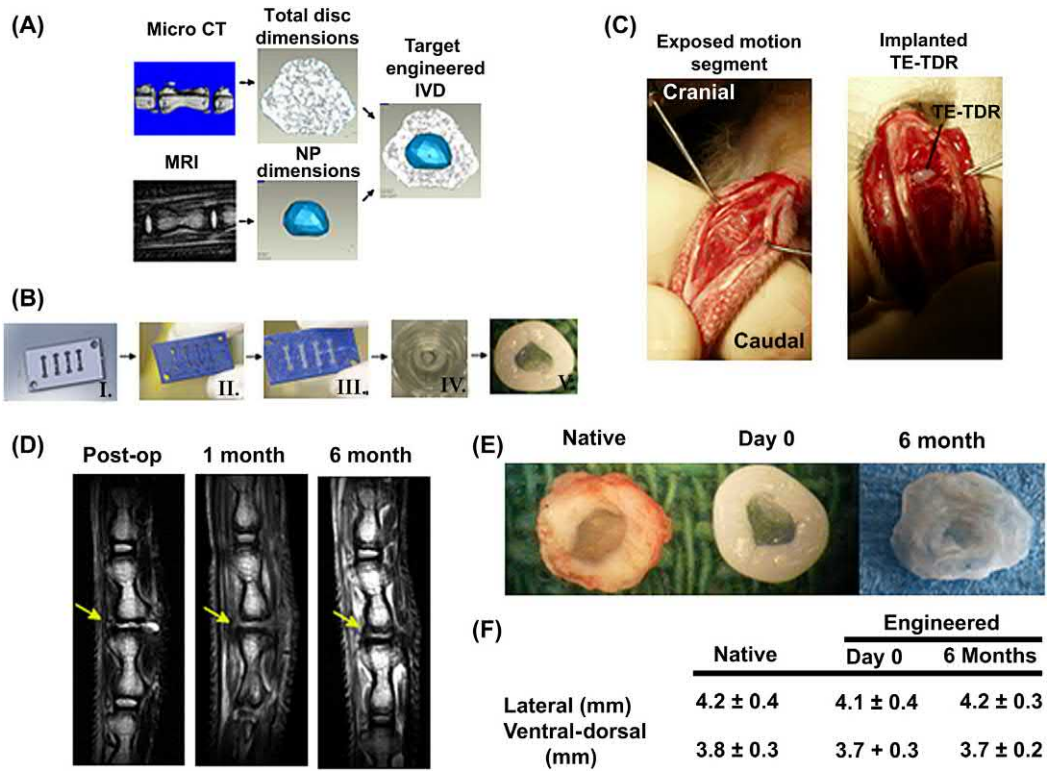

FIGURE 51.7 Anatomical composite TE-IVD, designed from MRI and CT, survives in disk space for 6 months.
(A) CT and MRI design procedure for obtaining TE-IVD dimensions. (B) Fabrication of TE-IVD. (I) NP dimensions used to design injection molds via computer-aided design. (II) Injection mold 3-D printed out of acrylonitrile butadiene styrene plastic. (III) Cell-seeded alginate was injected into mold, removed, (IV) placed in center of 24 well plate, and cell-seeded collagen was poured around alginate NP. (V) After 2 weeks of culture, cell-seeded collagen contracts around the NP to form composite TE-IVD. (C) Intraoperative images showing exposed caudal 3/4 disk space and implanted TE-IVD. (D) T2-weighted MRI of implanted disk space (marked by *yellow arrows*) and adjacent native levels immediately postoperative, at 1 month, and 6 months after implantation. (E) History of TE-IVD in native disk space. Intraoperative photo showing explanted native IVD next to the TE-IVD (day 0) that was implanted in its place and TE-IVD after being implanted into native disk space for 6 months. (F) Size of engineered IVD compared to native IVD. Measurements were taken along the lateral and ventral−dorsal planes of the engineered and native IVD. Engineered IVD measurements were taken at day 0 prior to implantation ($n = 12$) and compared to explanted native disks ($n = 12$). Engineered IVD measurements were also taken after 6 months of implantation ($n = 12$). *IVD*, Intervertebral disk; *MRI*, magnetic resonance imaging; *NP*, nucleus pulposus; *TE*, tissue-engineered. *Reprinted with permission from Bowles, et al. PNAS 2011.*

derived from porcine, lapine, or rodent tissues may be largely notochordal. Further, the notochordal or mesenchymal original of cells from canine sources is known to vary by breed. These phenotypic differences add an additional and unique complicating factor for investigators studying preclinical models for IVD tissue regeneration.

Given the very limited availability of native IVD cells that can be effectively harvested for tissue engineering, there has been an interest in using other cells as sources for these efforts. The primary target for other sources has been stem cells derived from sources such as bone marrow [110], embryonic cell lines [111], and adipose tissue [112]. A major challenge in this approach has been the development of methods to guide the development of stem cells toward phenotypes found in the IVD (see the next section). This has been attempted through manipulation of the culture medium and gas conditions [113], as well as coculture with primary cells from the IVD [114,115]. The more recent development of induced pluripotent stem cells has also provided the possibility for an additional cell source for cell delivery to treat musculoskeletal disorders [116]. In comparison to the use of adult primary disk cells derived from often pathological or degenerated IVDs, the use of autologous mesenchymal stem cells (MSCs) or progenitor cells may be most promising to the future of ex vivo tissue-engineering strategies that rely upon cell supplementation.

Cell therapy preclinical studies

If the local environment within the IVD is conducive to the survival of cells, direct cell supplementation without biomaterial scaffolds may hold promise for IVD repair. This strategy has been pursued by several groups preclinically and clinically, using either IVD cells, chondrocyte-like cells, or progenitor cells.

In the first reported work of disk cell supplementation, NP tissues from the rat were inserted following removal of nucleus tissue and showed some beneficial effects in inhibiting the degenerative IVD changes of nucleotomy [117]. Since then, several preclinical studies have reported the benefits of cell supplementation in delaying disk degeneration. These studies include mouse, rat, rabbit, canine, pig, goat, and sheep animal models [118].

In studies of cell therapy, three primary types of cells have been utilized: disk cells, chondrocytes, and stem cells [118]. Ideally, cells derived from native or native-like tissue (i.e., cartilage) would be used as they are better suited to disk environments they are implanted into, but there are limitations in using these cells, as it is relatively difficult to obtain sufficient cell numbers and cell activity may be impaired in cells derived from degenerated tissue. To mitigate issues of cell number, some studies have focused on delivery of cells through delivery of allograft tissues based on the concept that less cells are lost in processing and that preservation of ECM is an equally important criteria for regeneration [80,107]. To mitigate issues of impaired cell activity, some studies have focused on using techniques for cell reactivation [119]. These approaches were shown to be effective in delaying some degenerative features such as the loss of disk architecture following reinsertion of the "activated" cells in a rabbit model. Overall studies with disk cells and chondrocytes have shown positive effects and safety, which has allowed them to move forward into clinical trials as discussed in the next subsection [120].

With challenges associated with obtaining disk or disk-like cells, there has also been a focus on developing stem cell therapies for disk regeneration [121,122]. There have been several in vivo studies that have demonstrated efficacy; therefore these therapies have also moved forward to clinical trials as described in the next subsection. Still, there are concerns regarding stem cell survival in the harsh disk environment which questions the longevity of these stem cell therapies [123]. Therefore current preclinical work is focused on enhancing survival and regenerative capability of stem cells in these environments. This has been investigated through preconditioning, genetic engineering, and biomaterials. An example regarding preconditioning shown by Peck et al. demonstrated hypoxic expansion of stem cells that enhance their survival in low oxygen and low nutrient environments, like that of the disk [124]. Biomaterials in general have been considered a necessity for cell delivery to prevent osteophyte formation, but many have also been utilizing them to improve therapeutic outcomes of cell therapy [73]. For example, Tsaryk et al. demonstrated that a collagen-hyaluronic acid interpenetrating network with transforming growth factor (TGF) β3 loaded gelatin microspheres could perform successful cell delivery and induce differentiation of MSCs in vivo in mice [125]. Gene therapies have investigated enhancing cell inflammatory resistance and/or differentiation of stem cells ex vivo [126,127]. Enhancing resistance to inflammation is also applicable to nonstem cell therapies and has been applied to disk cells in vitro [128]. These studies hope to provide methods to overall further improve all cell therapies and increase their longevity and rate of success.

Cell therapy clinical studies

The first study clinically investigating implantation of disk cells began in 2002 with a prospective, controlled, multicenter study undertaken to compare autologous disk cell transplantation plus discectomy against discectomy alone [129]. Since then, a total of 14 clinical trials of cell therapies have been published and the results of these trials are reviewed in further detail elsewhere [120]. In addition, several cell therapy clinical trials are currently underway and the results of these studies are pending. Overall the results of clinical trials of cell therapies, utilizing cells derived from multiple tissue sources, including disk cells, chondrocytes, platelet-rich plasma (PRP), and stem cells, have shown promising therapeutic potential in initial studies.

One approach for IVD cell therapy has been the implantation of autologous IVD chondrocytes [129,130]. In these clinical trials, patients receiving cell transplantation reported improved pain and reduced disability. In addition, the IVDs of patients receiving cell implantation showed significant improvement in disk hydration when compared to the IVDs of patients in the discectomy group. However, disk height was found to be similar in patients receiving cell transplantation and those in the discectomy group. This clinical study led to the development of at least one product termed ADCT, or autologous disk chondrocyte transplantation with or without a hydrogel carrier (NOVOCART), which has been widely used and approved in Germany since 2008. Utilizing a similar approach, clinical trials have demonstrated that patients receiving intradiscal implantation of allogenic chondrocytes reported reduced pain, improved disability scores, and improved flexibility when compared to patients undergoing discectomy [131].

In addition to chondrocytes, clinical trials have tested the implantation of IVD cells as cell-therapy treatment strategy. Mochida et al. demonstrated that patients receiving autologous NP cell implantation reported improved pain and disability scores. In addition, disk height and disk hydration were maintained, and Pfirrmann's classification of disk degeneration was improved in patients receiving intradiscal IVD cell implantation [132]. Overall, these clinical trials have established the safety of intradiscal chondrocyte and IVD cell implantation and the initial findings of these clinical trials reported improvements in

several important patient outcomes, including pain and disability scores. Further clinical trials are required to determine the efficacy of these cell implantation strategies for the treatment of painful disk degeneration.

Due to the inherent properties of MSCs, the implantation of MSCs harvested from a patient's own bone marrow [133–138] or adipose tissue [139] for the treatment of disk degeneration and/or disk herniation has been tested in recent clinical trials. In clinical trials utilizing MSCs harvested from autologous bone marrow, patients receiving intradiscal transplantation of autologous MSC reported decreased pain and improved disability scores at up to 6 years following procedures [133–138]. In addition, the IVDs of patients receiving cell transplantation demonstrated reduced disk bulge size, maintained disk height, and maintained or improved the level of disk degeneration (Pfirrmann's classification).

In addition to bone marrow MSCs the ability of intradiscal transplantation of adipose-derived MSCs to regulate disk degeneration has been tested in recent clinical trials [139]. In these studies, patients receiving autologous adipose derived MSC implantation reported improved pain and disability scores. In addition, the disks of patients receiving cell transplantation exhibited maintained disk height and maintained levels of degeneration. Together, these clinical studies have demonstrated the safety of MSC cell transplantation to the pathological disk, and the ability of MSC implantation to regulate several key outcomes for patients with disk degeneration and/or herniation.

Growth factors and other biologics for intervertebral disk regeneration

In vitro studies

Disk cells modulate their activity by a variety of substances, including cytokines, growth factors, enzymes, and enzyme inhibitors in a paracrine and/or autocrine fashion [140]. Tissue-engineering approaches to disk regeneration have been based on attempts to upregulate important matrix proteins (e.g., aggrecan) or to downregulate proinflammatory cytokines [e.g., interleukin-1 (IL-1); tumor necrosis factor-α (TNF-α)] [141–147] and matrix-degrading enzymes [e.g., matrix metalloproteinases (MMPs); aggrecanases] [148–151]. The delivery of these modulating biologic agents, with and without scaffolds and/or through cell transplantation, has been the subject of many years of efforts in tissue engineering. In vitro studies have shown that the rate of matrix synthesis or gene expression for matrix proteins, principally proteoglycan or collagen, can be increased several-fold in IVD cells in the presence of one or a combination of these growth factors: supplemental TGF-β [152–157] and its subtype TGF-β3 [158–160], osteogenic protein-1 (OP-1; otherwise known as BMP-7) [58,161–163], BMP-2 [164–166], BMP-12 [166], growth and differentiation factor-5 (GDF-5) [167–169], epidermal growth factor (EGF) [170], connective tissue growth factor (CTGF) [171], or insulin-like growth factor-1 (IGF-1) [172,173]. Other studies have demonstrated the potential of these growth factors, as well as platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF), to reduce cell apoptosis [174] and to promote cell proliferation [175,176]. Several recent studies examined the effects of the in vitro costimulation of IVD disk cells by two or more growth factors. In micromass cultures of fetal outer annulus cells, TGF-β1 and IGF-1, both alone and in combination, stimulated the synthesis of sulfated GAGs, and collagen types I and II. While TGF-β1 and BMP-2 each had strong anabolic effects on NP cells cultured in an atelocollagen scaffold, there were no additive or synergistic effects on cell proliferation and matrix synthesis. Autologous PRP, which contains a variable mixture of growth factors, including TGF-β1, PDGF, and IGF-1, has also been shown to be an effective stimulator of cell proliferation, proteoglycan, and collagen synthesis, as well as proteoglycan accumulation, when added to porcine IVD cell cultures in vitro. Other studies examined the effect of PRP on bovine AF cells and found that it increased aggrecan and type-II collagen production and also decreased the catabolic protein MMP-1 [177,178]. In human NP cells, PRP containing 1 ng/mL TGF-β1 induced cell proliferation and differentiation and promoted the in vitro formation of tissue-engineered NP as indicated by cell morphology, mRNA expression profile, and GAG accumulation [179] (Table 51.3).

In a different approach, supplementation of IVD cell cultures with a naturally occurring antiinflammatory molecule, IL-1 receptor antagonist (rhIL-1Ra), has been shown to inhibit the downregulation of biosynthesis induced by the proinflammatory cytokine, IL-1 [176]. Pretreatment of NP cells with both IL-1Ra and a fusion protein between IL-1Ra and an ELP reduced the expression of MMP-3 and the aggrecanase, a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4), when the cells were subsequently treated with IL-1 [203]. The in vitro application of rhIL-1Ra to degenerated human disk tissues reduced levels of enzymes, MMP-3, -7, and -13, implicated in the degradation of the IVD [204]. The incubation of herniated human disk tissues with IL-1Ra or an antibody inhibitor of TNF both decreased levels of active MMP-3, indicating that these inhibitors may have an effect on the resorption of herniated disks [205]. The inhibition of p38 mitogen-activated protein kinase in IL-1-activated NP cells reduced the production of factors associated with the catabolic effects of IL-1 and TNF-α [206]. An extension of that study revealed that the IL-1 upregulation of gene

TABLE 51.3 Representative gene, growth factor, and cell therapy studies.

Factor (s)	Cell type and source	Delivery	Results	Year	Reference
bFGF	Bovine IVD cells	rhFGF (5 ng/mL) for 24 h	Induced cell proliferation of quiescent cells	2007	[173]
BMP-2	Rat NP cells	rhBMP-2 (100, 1000 ng/mL)	Increased collagen and sGAGs, as well as expression of type-II collagen, aggrecan, and Sox9 mRNA	2003	[165]
	Human IVD cells	rhBMP-2 (2.5–2000 ng/mL) for 3 weeks	Upregulation of aggrecan and type-I/type-II collagen mRNA. Increased proteoglycan synthesis	2003	[164]
	Human IVD cells	rhBMP-2 (25–300 ng/mL) for 2 days	Increased collagen and proteoglycans in NP cells only	2008	[166]
BMP-12	Human IVD cells	rhBMP-12 (25–100 ng/mL) for 2 days	Increased collagen and proteoglycans in NP cells only	2008	[166]
	Human NP and AF cells	Adenovirus	Increased cell proliferation and matrix protein synthesis in NP and AF cells	2008	[166]
CTGF	Rhesus monkey IVD cells	Adeno-associated virus	Increased proteoglycan and type-II collagen synthesis	2010	[171]
EGF	Canine IVD cells	EGF (1 ng/mL) for 48 h	Increased cell proliferation and stimulation of matrix synthesis	1991	[170]
GDF-5	Bovine IVD cells	rhGDF-5 (100, 200 ng/mL) for 21 days	Increased cell proliferation, proteoglycans, and collagen in both NP and AF cells	2006	[167]
	Mouse IVD cells	Mouse GDF-5 (1–100 ng/mL) for 9 days	Upregulation of aggrecan and type-II collagen mRNA	2004	[168]
	Mouse IVD cells	Gene therapy	Increased type-II collagen and aggrecan mRNA expression	2007	[169]
IGF-I	Bovine IVD cells	rhIGF-I (100 ng/mL) for 24 h	Induced cell proliferation of quiescent cells	2007	[173]
	Bovine NP cells	IGF-I (0.1–1000 ng/mL)	Increased proteoglycan synthesis	1996	[172]
OP-1	Human IVD cells	rhOP-1 (100, 200 ng/mL) for 21 days	Prevention of decreased cell number Increased proteoglycan synthesis and accumulation	2007	[163]
	Rabbit IVD cells	rhOP-1 (100, 200 ng/mL) for 72 h	Increased cell proliferation, total proteoglycan, and collagen	2006	[60]
	Rabbit IVD cells	OP-1 (200 ng/mL) for 21 days	Rapid recovery of proteoglycans and collagens which were lost as a result of IL-1α treatment	2002	[61]
	Rabbit IVD cells	OP-1 (200 ng/mL) for 21 days	More rapid recovery of proteoglycans and stimulated matrix repair following chemonucleolysis with chondroitinase ABC	2005	[62]
PDGF	Bovine IVD cells	rhPDGF-BB (10 ng/mL) for 24 h	Induced cell proliferation of quiescent cells	2007	[73]
TGF-β	Human NP cells	TGF-β1 (10 ng/mL) for 6 days	Increased aggrecan, type-I and type-II collagen, and Sox9 mRNA expression	2013	[53]
	Rat AF cells	TGF-β1 (5 ng/mL) for 5 days	Increased GAG output and type-I/type-II collagen	2011	[56]

(*Continued*)

TABLE 51.3 (Continued)

Factor (s)	Cell type and source	Delivery	Results	Year	Reference
	Human AF cells	TGF-β1 (5 ng/mL) for 7 days	Stimulated ECM production. Increased production of sulfated proteoglycans	2010	[51]
	Human IVD cells	TGF-β1 (0.25–5.0 ng/mL) for 10 days	Enhanced cell proliferation. Increased biglycan production	1997	[52]
	Rat IVD	TGF-β3 (10 ng/mL) for 1 week	Preservation of disk morphology and architecture. Prevention of cell death. Increased expression of ECM genes and sulfated proteoglycans	2006	[57]
	Human NP cells	TGF-β3 (10 ng/mL) for 28 days	Increased collagen and aggrecan gene expression. Production of proteoglycan- and type-II collagen-rich matrix	2009	[58]
	Human NP cells	TGF-β3 (10 ng/mL) for 7 days	Increased cell proliferation. Exhibition of an anticatabolic gene expression profile	2012	[59]
TGF-β, BMP-2	Rabbit NP cells	rTGF-β (10 ng/mL)rBMP-2 (100 ng/mL)	Increased cell proliferation and proteoglycan synthesis Upregulation of aggrecan, type-I/type-II collagen mRNA	2012	[75]
PRP, TGF-β	Human NP cells	PRP containing TGF-β (1 ng/mL) for 7 days	Upregulated Sox9, type-II collagen, and aggrecan mRNA Increased GAG accumulation	2006	[79]
IGF-1, βMP-2, TGF-β	Human IVD cells	Adenovirus	Increased proteoglycan synthesis	2008	[54]
PRP	Bovine AF cells	25%–50% PRP for 4 days	Increased GAG synthesis and cell number	2014	[77]
	Bovine AF cells	PRP for 24 h	Increased type-II collagen and aggrecan. Decreased MMP-1	2016	[78]
	Porcine IVD cells	PRP for 72 h	Upregulated proteoglycan, collagen synthesis, and cell proliferation	2006	[76]
Factor	**Animal model/ clinical trial**	**Delivery**	**Results**	**Year**	**Reference**
BMP-2	Rabbit (annular tear)	rhBMP-2 (100 μg in 100 μL)	More frequent and severe degeneration. Increased vascularity and fibroblast proliferation	2007	[180]
GDF-5	Rat (compression)	GDF-5 (8 ng in 8 μL)	Expansion of inner annular fibrochondrocytes into nucleus. Cells expressed aggrecan and type-II collagen mRNA. Increased disk height	2004	[181]
	Rabbit (puncture)	rhGDF-5 (0.01–100 μg)	Restoration of disk height. Improved MRI and histologic grading scores	2006	[167]
	Mouse (puncture)	Adenovirus	Increased disk height. Improved GAG retention	2010	[182]
	Phase I/II clinical trial ($n = 32$)	rhGDF-5 (0.25, 1.0 mg)	Improved patient-reported pain scores and a decrease in pain-related disability	2016	[183]
	Phase II clinical trial ($n = 24$)	rhGDF-5 (1.0, 2.0 mg)	Failed to show significant improvements over placebo for Oswestry Disability Index, Pain Visual Analog Scale, and quality of life	2016	[184]
GDF-6	Sheep (annular puncture)	GDF-6 (300 μg in 70 μL)	Greater disk hydration retention. Reversed or arrested loss of ECM proteins	2009	[101]
	Rabbit (annular puncture)	GDF-6 (100 μg in 10 μL)	Partial restoration of disk height	2018	[185]
HGF	Rat (annular pucture)	rhHGF (10 ng in 2 μL)	Increased type-II collagen in the NP. Retardation of disk degeneration as observed by MRI	2013	[186]

(*Continued*)

TABLE 51.3 (Continued)

Factor	Animal model/ clinical trial	Delivery	Results	Year	Reference
IGF-1	Rat (compression)	IGF-1 (8 ng in 8 μL)	Early, transient increase in cell density and cell proliferation	2004	[181]
OP-1	Rabbit (normal)	rhOP-1 (2 μg in 10 μL)	Increased disk height and proteoglycan content	2005	[187]
	Rat (chronic compression)	OP-1 (200 ng in 1 μL)	Prevention of mechanical hyperalgesia. Increased ECM	2005	[188]
	Rabbit (puncture)	rhOP-1 (100 μg in 10 μL)	Increased water content in NP. Increased proteoglycan content in NP and AF. Lower degeneration grades	2006	[189]
	Rabbit (annular puncture)	OP-1 (100 μg in 10 μL)	Restored biomechanical properties of degenerated IVDs. Higher IVD elastic and viscous moduli. Increased proteoglycan and collagen content	2006	[190]
	Rabbit (chemonucleolysis)	rhOP-1 (100 μg in 10 μL)	Increased disk height; higher proteoglycan content	2007	[191]
	Rat (chronic compression)	OP-1 (200 ng in 1 μL)	Restoration of normal disk morphology. Increased Safranin O staining in NP. Extended ECM. Reduced presence of catabolic proteins	2007	[192]
PDGF	Rabbit (annular puncture)	PDGF-BB (1 ng/μL/disk)	Prevention of apoptosis and collagen-3 matrix deposition. Decreased degeneration. Increased compressive strength	2016	[193]
TGF-β	Rabbit	Adenovirus	Increased proteoglycan synthesis	1999	[194]
	Rat (compression)	TGF-β1 (1.6 ng in 8 μL)	Increased population of annular fibrochondrocytes. Increased matrix synthesis	2004	[181]
	Phase I/IIa clinical trial ($n = 50$)	YH14618 (1–6 mg/disk)	Improvements in Oswestry Disability Index and Pain Visual Analog Scale. No significant changes in disk height or MRI scores	2015	[195]
	Phase IIb clinical trial	YH14618	Trial halted as YH14618 failed to show anticipated benefits compared to placebo. Another phase-II clinical trial is anticipated	2016	[184]
PRP	Rabbit (nucleotomy)	PRP in gelatin hydrogel microspheres	Decrease in degeneration, increased proteoglycan in NP and inner AF	2007	[196]
	Rabbit (annular puncture)	PRP in gelatin hydrogel microspheres	Greater disk height. Preservation of water content. Higher mRNA expression of proteoglycan core protein and type-II collagen. Fewer apoptotic cells in NP	2009	[197]
	Rabbit (annular puncture)	PRP releasate (20 μL)	Restoration of disk height. Higher number of chondrocyte-like cells	2012	[198]
	Human; prospective trial ($n = 22$)	PRP (1.5 mL)	Improved patient-reported pain scores and a decrease in pain-related disability	2015	[199]
	Human; preliminary assessment ($n = 6$)	PRP (2 mL)	Improved patient-reported pain scores and increased patient function	2015	[200]
	Human (clinical study; $n = 29$)	PRP (1–2 mL)	Improvements in pain and function through 2 years of follow-up	2016	[201,202]

AF, annulus fibrosus; *bFGF*, basic fibroblast growth factor; *BMP*, bone morphogenetic protein; *CTGF*, connective tissue growth factor; *ECM*, extracellular matrix; *EGF*, epidermal growth factor; *GAG*, glycosaminoglycan; *GDF*, growth and differentiation factor; *IGF*, insulin-like growth factor; *IL*, interleukin; *IVD*, intervertebral disk; *MMP*, matrix metalloproteinase; *MRI*, magnetic resonance imaging; *NP*, nucleus pulposus; *OP*, osteogenic protein; *PDGF*, platelet-derived growth factor; *PRP*, platelet-rich plasma; *TGF*, transforming growth factor; *HGF*, Hepatocyte Growth Factor.

expression of MMP-3, IL-1, and IL-6 was reduced, while the IL-1 downregulation of matrix protein gene expression and proteoglycan synthesis was reversed in NP cells [206].

These above in vitro studies illustrate that both stimulatory factors, as well as antiinflammatory or anticatabolic factors, may be considered for therapeutic purposes in IVD regeneration. Overall, the potential for biologics to assist in matrix regeneration through controlling both cell metabolism and cell number has been established and paved the way for more recent studies evaluating these biologics in vivo.

In vivo studies: growth factors

Protein injection into the disk space is relatively simple and practical and has been the most widely studied of all approaches for delivery of growth factors and biologics for IVD regeneration. Walsh et al. reported the in vivo effect of single or multiple injections of several growth factors, including bFGF, GDF-5, IGF-1, or TGF-β, in mouse caudal disks with degeneration induced by static compression [181]. Fibrochondrocyte aggregates were observed at 4 weeks in the nucleus of disks that received a single injection of GDF-5 and multiple injections of TGF-β, while a single injection of IGF-1 may have elicited an early, transient response at 1 week and bFGF treatment had little effect. A statistically significant increase in disk height 4 weeks after GDF-5 treatment was also reported and the authors suggested that GDF-5 and TGF-β are mitogens for annular chondrocytes.

A single intradiscal administration of recombinant human OP-1 (rhOP-1) into normal rabbit disks in vivo resulted in increased disk height and proteoglycan content in the NP regions in comparison to a saline injection control group [187]. Other studies used a rabbit model of disk degeneration caused by needle puncture of the AF and showed that an injection of rhOP-1 (100 μg/disk) restored disk height, structural change, and mechanical properties [189,190]. In another rabbit model of disk degeneration a single injection of rhOP-1 (100 μg/disk) significantly reversed the decrease in disk height following chondroitinase ABC chemonucleolysis; the reversal was sustained for up to 16 weeks and resulted in a significantly higher proteoglycan content compared to the vehicle control group [191]. The in vivo efficacy of rhOP-1 has been confirmed in a rat model of chronic compression of tail IVDs; under continuous compression load the injection of rhOP-1 resulted in an increase of the ECM, as observed by histology, in the degenerated disks [188]. An extension of that study confirmed that a direct injection of OP-1 into the chronically compressed rat tail IVDs induced anabolic and anticatabolic activities, as documented by reduced immunostaining for aggrecanase, MMP-13, TNF-α, and IL-1β [192].

The effectiveness of direct protein delivery was also confirmed in experiments using rhGDF-5, where a single injection of rhGDF-5 resulted in the restoration of disk height, and improvements in MRI and histological grading scores in the rabbit annular puncture model of disk degeneration [167]. In this model a single injection of rhGDF-5 significantly suppressed the mRNA expression of cytokines (IL-1β, IL-6, TNF-α), catabolic enzymes (ADAMTS4, cyclooxygenase-2), and pain-related molecules [nerve growth factor (NGF), vascular EGF] by punctured disks. The benefits of increased levels of GDF-5 on IVD regeneration have also been explored by a more indirect mechanism. In a mouse annular puncture model, GDF-5 overexpression vectors delivered via adenovirus increased disk height and improved GAG retention [182]. However, despite the apparent success of intradiscal injection of rhGDF-5 in animal models, the results have not been able to be replicated in clinical trials. An initial combined phase I/II clinical trial with 32 participants showed promising results as patients reported improved pain scores and a decrease in pain-related disability [183], but a double-blind placebo-controlled phase II clinical trial revealed no significant improvements over placebo in pain, disability, or quality of life [184].

In a rabbit annular stab model of disk degeneration, an intradiscal injection of BMP-2 resulted in degenerative changes that were more frequent and severe than with the saline control, and promoted hypervascularity, and fibroblast proliferation but had no effect on new bone formation or fusion of the IVD after 12 weeks; the authors suggest that rhBMP-2 may be involved with a response to injury after an annular tear [180]. In an annular stab ovine large animal model of disk degeneration the simultaneous injection of recombinant human GDF-6 (rhGDF-6; also known as BMP-13), which plays a role in spinal column development and promotes the expression of chondrogenic marker genes in a variety of cell types, was protective against loss of disk height and cells from the disk, and resulted in increased levels of disk ECM proteins compared to annular stab alone [101]. In a rabbit annular puncture model, intradiscal injection of GDF-6 (10 or 100 μg/disk in PBS) 4 weeks after degeneration was initiated resulted in a partial restoration of disk height and an improvement of MRI degeneration grades 12 weeks later [185].

Intradiscal injections of rhPDGF-BB in a thiol-modified hyaluronic acid hydrogel in a rabbit annular puncture model showed that PDGF prevented cell apoptosis and the deposition of matrix containing type-III collagen 4 weeks after treatment [193]. When examined 8 weeks after treatment, PDGF-treated degenerated IVDs had significantly higher MRI indices, reduced degeneration, and an increase in compressive strength to failure.

The in vivo application of PRP-impregnated gelation hydrogel microspheres (PRP-GHMs) has been evaluated

in a nucleotomy rabbit model of disk degeneration. Histological and immunohistochemical studies revealed that the structure of the IVD and the accumulation of proteoglycans were preserved by the injection of PRP-GHMs, but not in PRP alone and saline controls [196]. An extension of these studies in this model reported that

1. disk height and water content were preserved in PRP-GHMs-treated IVDs in MR images,
2. the mRNA expression of proteoglycan core protein and type II collagen was significantly higher, and
3. the number of apoptotic cells in the NP was lower after PRP-GHM treatment compared with other treatment groups (PRP-saline, saline-GHM, puncture only) [197].

PRP treatments continue to show promise in human trials for treating degenerative disk disease. In preliminary human assessments and clinical trials, patients receiving PRP injections have reported modest improvements in pain and function as measured by the pain visual analog scale (VAS) and the Oswestry Disability Index (ODI) even up to 2 years after receiving the treatment [199–202].

Aberrant TGF-β signaling has been implicated in matrix degradation and subsequent IVD degeneration [195,207,208]. Increasing the presence of TGF-β by its overexpression or by delivering recombinant protein directly to the disk has shown promise in multiple animal models by exhibiting increased matrix synthesis and an increase in annular fibrochondrocytes [181,194]. However, TGF-β is highly expressed in patients with DDD and has also been shown to increase the presence of catabolic proteins such as aggrecanase and MMP through a secondary signaling pathway via the ALK1 receptor that results in the phosphorylation of Smad 1/5/8 [207,208]. This concept gave rise to another unique approach to modulating TGF-β signaling that employs a peptide known as YH14618. This peptide binds to TGF-β1, thus causing its preferential binding to specific receptor heterodimers and resulting in a reduction in downstream Smad 1/5/8 phosphorylation [184,195,209]. The outcome of these processes is an increase in the synthesis of ECM components and a reduction in TGF-β-induced NGF expression [195]. In 2015 YH14618 completed a combined Phase I/II clinical trial with 50 participants that showed improvements in both VAS and ODI, but no significant changes in disk height or MRI scores. The following year, a Phase IIb trial was halted as YH14618 failed to show the anticipated benefits as compared to the placebo group [184]. Despite the setback of the failed clinical trial, YH14618 was recently licensed to a separate company and another clinical trial is expected to begin soon.

In vivo studies: other biologics

LinkN is an in vivo proteolytic degradation byproduct of proteoglycan aggregate stabilizing link protein. The effect of the intradiscal administration of the synthetic peptide of LinkN, which stimulates proteoglycan and collagen synthesis of IVD cells in vitro, was evaluated in the rabbit anular puncture model of disk degeneration and was shown to partially restore disk height, increase anabolic ECM gene expression, and reduce catabolic gene expression [156]. In a recent study in a large animal model of disk degeneration, the disk architecture and mechanical properties of surgically denucleated porcine (minipig) IVDs injected with a fibrin sealant, which promotes IVD cell proliferation and matrix synthesis, were preserved [210]. In addition, fibrin also inhibited fibrosis of the NP, increased proteoglycan synthesis, reduced secretion of proinflammatory cytokines, and increased the synthesis of the proresolution factors, TGF-β, and IL-4. Nasto et al. have recently demonstrated that inhibition of NFκB activity via intraperitoneal injection of Nemo Binding Domain peptide reduced proteoglycan loss in disk degeneration observed in a mouse model of accelerated aging [211].

The therapeutic use of PRP treatments for the management of chronic low back pain has recently been tested in multiple preclinical [196–198,212–217] and clinical trials [199,201,218,219]. The results of these studies have been reviewed in detail elsewhere [220]. PRP is a concentrated, autologous blood product containing platelets, as well as natural concentrations of cytokines and growth factors [221,222]. The recent success of PRP therapies in the treatment of tendinopathies [223] and osteoarthritis [224] have led to increased interest in PRP treatment for low back pain and disk degeneration. In small animal models of disk degeneration, disks receiving intradiscal PRP injection have demonstrated decreased disk degeneration [196,216], increased disk height, increased disk hydration [197], and improved of disk appearance and signal in MRI imaging [216]. In clinical trials, patients receiving intradiscal PRP injection for the treatment of painful disk generation reported reduced pain [199,201,218] and improved disability scores when compared to patients in discectomy group. While findings from these clinical trials are promising, further clinical trials will be required to establish a standardized PRP treatment for painful disk degeneration.

Gene therapy for intervertebral disk regeneration

While the studies described above illustrate a range of proteins considered as possible therapies for IVD regeneration, it is important to consider the unavoidable limitations of protein delivery to the disk space. Issues such as protein half-life or solubility, the need for a proper carrier, need to preserve mechanical environment or cell numbers, and/or the presence of inhibitors are all factors that can be

expected to affect the therapeutic efficacy of protein delivery in vivo. A consideration for the use of recombinant protein therapies is also cost, as some disk pathologies and the need to inhibit disk degeneration may be chronic in nature or require multiple treatments. Gene therapy has been advocated as a therapeutic alternative for the delivery of biologics in disk regeneration [225]. DNAs that encode specific proteins may be delivered into the cells by viral or nonviral transfection, with the result that these cells produce proteins to, theoretically, prolong the duration of action. In this section, we discuss multiple methods of gene therapy that have been performed for regeneration and inhibition of degeneration of the IVD.

Gene transfer studies: viral

Adenoviral vectors often possess high titers and infectivity and are able to infect nondividing cells such as IVD cells. Adenoviral-mediated gene transfer to human IVD cells has been shown to be efficient and to produce transcripts across nondegenerative to degenerative cell types [226,227]. Studies have demonstrated an ability to increase expression of anabolic proteins such as Sox9, GDF-5, and LMP-1 in disk cells and have anabolic effects including increased proteoglycan synthesis [227–229]. The feasibility of using direct in vivo adenoviral-mediated gene transfer to disk cells has also been demonstrated for anabolic proteins such as GDF-5, TGF-β1, and LMP-1 and shown to exert a biological effect on biosynthesis, often for several weeks [182,194,228].

In addition to upregulation of anabolic factors, inhibition of catabolic processes has also been studied using gene therapy for IVD regeneration. Wallach et al. reported that gene transfer of the tissue inhibitor of metalloproteinase-1 (TIMP-1), an inhibitor of catabolic enzymes, can increase proteoglycan accumulation within pellet cultures of human IVD cells [230]. Le Maitre reported that human disk cells infected with Ad-IL-1 receptor antagonist (Ad/IL-1Ra) were resistant to IL-1 [231]. When in vitro–infected cells were injected into disk explants in vitro, IL-1 receptor antagonist protein expression was also increased and maintained for the 2-week time period investigated.

There are significant concerns about adenoviral vector use clinically, however, that may include significant toxicity when used in spinal applications [232,233]. These concerns have led investigators to begin consideration of adeno-associated viral vectors (AAVs) which are known to be less immunogenic [234]. Studies have been performed with AAV to deliver genes previously delivered with adenovirus, including the delivery of Sox9, TIMP1, TGF-β1 in vivo [235–237]. All of these studies demonstrated measurements that indicated delayed disk degeneration. Therefore even though AAV systems have a disadvantage in that they induce lower levels of protein expression and the expression of transferred genes is more delayed, they can still mediate gene transfer with desired effects in the IVD [238].

Adenoviral vectors and AAV are a form of transient gene transfer as genes do not integrate into the genome. Therefore to have more long-term effects lentivirus has also been considered for IVD gene therapy [239]. Lentivirus has successfully been used to deliver anabolic genes, and inhibitors of catabolic processes in vivo. In a study by Yue et al., it was demonstrated that delivery of Survivin, an antiapoptotic gene, by lentivirus in vivo into a rabbit animal model reduces disk cell apoptosis [240]. Yue et al. also performed an additional study where they multiplexed lentivirus-based Survivin overexpression with TGFβ3 and TIMP1 overexpression. In this study, they demonstrated improved matrix composition in addition to decreased apoptosis [241]. The other in vivo lentiviral gene delivery study done to date investigated the delivery of TGFβ3 and TIMP1 along with CTGF [242]. This study by Liu et al. demonstrated that degeneration characterized by MRI was ameliorated, and type II collagen and aggrecan expression levels were significantly higher than puncture control for up to 20 weeks after treatment. Overall positive effects of lentivirus-mediated gene delivery have been demonstrated by these studies and some assessment of safety has been performed [239], but more extensive studies of longevity and safety are needed. The main safety concerns associated with lentivirus are insertional mutagenesis which can have a carcinogenic effect if the insertion affects a gene associated with cell division. This has become less of a concern with new generations of lentivirus which are much safer [243]. In addition, recent clinical trials have utilized lentivirus for in vivo gene delivery for the treatment of Parkinson's and macular degeneration and have not observed adverse effects related to the lentivirus [244,245].

Gene transfer studies: nonviral

To avoid safety concerns found with viral gene transfer, several nonviral methods for direct gene transfer to cells have been proposed. Reports using microbubble-enhanced ultrasound gene therapy [246] and a "gene gun" method [247,248] have shown that the introduction of a marker or growth factor gene could be accomplished and provides sustained gene expression without the need for viral vectors. These methods still demonstrated a low efficiency for the transfection of plasmids (~8%–14%). With a need to improve nonviral gene transfer efficiency, more recent research has focused on making this gene transfer more efficient. For example, in a study by Feng et al., nanofibrous spongy microspheres, which are a complex of hyperbranched polymers and DNA, demonstrated a

transfection efficiency of 32.4% into rat NP cells, and an ability to deliver NR4A1 (a fibrosis inhibitor) and have a therapeutic effect [249]. Another study by Feng et al. developed a cationic block copolymer gene delivery system that demonstrated an efficiency of 32.5% in vitro and therapeutic effects when used to deliver heme oxygenase-1 in vivo [250].

Endogenous gene regulation

Aside overexpressing therapeutic genes via exogenous gene delivery, there has also been an investigation of endogenous gene regulation. This regulation of endogenous genes in disks and disk cells has been performed through both viral and nonviral methods.

Nonviral methods have successfully been used to deliver and overexpress small transcripts for regulating gene expression, such as microRNAs and siRNAs. Regarding siRNA, both in vitro and in vivo have been performed and shown positive results [225]. Seki et al. have demonstrated a single injection of naked ADAMTS5 siRNA oligonucleotide demonstrated improved MRI scores and histological grade in a rabbit annular puncture model [251]. Banala et al. simultaneously delivered siRNA against caspase-3 and ADAMTS5 via lipoplexes in vivo and saw regenerative effects in histological analysis [252]. MicroRNA is a regulatory RNA that has more recently been discovered to play roles in disk degeneration by regulating genes associated with disk degeneration or disk tissue maintenance [253]. Delivery of microRNA mimics or microRNA inhibitors can be achieved nonvirally, with significant positive effects. For example, in a study by Sun et al., overexpression of miR-155 by lipofectamine-based transfection into rat NP cells significantly inhibited matrix degradation [254]. In a study by Zhang et al., overexpressing miR-140-5p using lipofectamine transfection inhibited liposaccharide-mediated inflammation in human NP cells [255]. An in vivo study by Ji et al. demonstrated that delivery of an oligo inhibiting miR-141, a microRNA, which mediates progression of disk degeneration using a commercial lipid−based delivery system, reduced histological scores and decreases in disk height in a mouse model of disk degeneration [256]. The microRNA field is still relatively new in IVD research, and there is still work to be done to evaluate if this nonviral microRNA delivery is feasible in vivo, but it holds promise in providing a gene therapy with a regenerative effect.

Viral methods have been utilized to deliver CRISPR/dCas9 systems to disk cells for endogenous gene regulation. To date, only two studies have utilized disk cells, both performing gene downregulation with the CRISPR/dCas9 system [128,257], and several have been performed in stem cells with the purpose of enhancing their regenerative effects [258]. Farhang et al. successfully downregulated TNFR1 expression via lentiviral delivery to human NP cells and demonstrated inhibition of apoptosis and catabolic gene expression under inflammatory stimulation [128]. Although it has not been performed in disk cells yet, these systems can also be used to upregulate endogenous genes [259]. This may be useful in future work for regulating genes mediating ECM expression (i.e., aggrecan) in a multiplex manner, which have been difficult to overexpress, especially simultaneously, due to size. In fact, this concept has already been demonstrated in adipose-derived stem cells [260]. Overall, these CRISPR/Cas9 systems hold promise in regulating the expression of specific genes to enhance the regenerative effects of either endogenous disk cells or exogenously delivered cells.

Gene therapy in summary

Overall, there are advantages and disadvantages to each gene therapy method discussed. With safety concerns associated with viral delivery, nonviral methods are preferred if possible. Though nonviral methods are still not as efficient as viral methods, but with a rise of ex vivo engineered cells being used for therapies, as seen by a number of in clinical trials in this area [261], this issue may not be as problematic. In addition, ex vivo gene transfer may be more desirable even if we eventually do achieve high efficiency with nonviral methods. This is due to the proximity of the disk target to the spinal cord. Significant harm can occur when there is due to failure to control delivery, as seen with paralysis in rabbits due to accidental gene delivery in the intradural space [233]. Overall, there is a lot of ongoing gene therapy research for IVD regeneration, and there is still more work to be done in determining the best approach to use clinically.

In vivo preclinical models for intervertebral disk regeneration and replacement

In order for tissue-engineered IVD regeneration and replacement strategies to reach the clinic, there are critical translational steps that must occur to bring technologies from bench to bedside. Most tissue-engineered concepts are first developed and tested through ex vivo and in vitro models, with promising technologies moving onto small then large in vivo animal models. There are many well-defined ex vivo and in vitro models used to develop and analyze regenerative strategies; however, less is known about effective in vivo animal models for studying IVD regeneration. A vast array of animals have been used for in vivo study of IVD degeneration and

regeneration ranging from sand rats to primates [64,99,262–268]. Some models are chosen because they naturally degenerate and simulate aging in humans, while others are induced to a degenerative state by using methods ranging from mechanical injuries to chemical digestion to long-term smoking. The ideal animal model will depend on the tissue-engineered strategy being used, where regeneration strategies typically are applied on degenerated IVDs, while whole IVD replacement studies may not require degeneration prior to implantation. Knowing how to use these models to advance the translational potential of tissue-engineered regeneration schemes is equally as important as the repair technology itself. This section will briefly describe current animal models used to study IVD degeneration and regeneration, but for additional reading material Singh et al. provide an excellent review.

When selecting an animal model for in vivo study, there are a variety of factors to consider depending on the type of regenerative strategy and the purpose of the study. Small animal models have been proven to be valuable tools for initially screening a variety of therapeutics for therapeutic efficacy and immune response but do not mimic the geometry or mechanical loads seen in humans IVDs [268]. Rat tail and lumbar spine models are popular choices for initial screening of novel tissue-engineered strategies for their ease of surgical access and well-defined degeneration progression. Degeneration is not a natural phenomenon in either rat tail or lumbar IVDs; however, it may be induced through mechanical injury, tail bending, axial compression, or tail suspension. Another commonly used small animal model for in vivo study is New Zealand white rabbits, whose lumbar IVDs have slightly larger geometry than rats and have been induced to degenerate through the above methods and chemical digestion, torsional injury, resection of bony processes, and fusion of adjacent IVDs.

Once a tissue-engineered therapy has been shown effective with minimal host response in a small animal model in vivo, large animal models are almost always necessary prior to translation to humans. Commonly used large animal models are sheep lumbar spines, goat lumbar spines, dog cervical and lumbar spines, pig lumbar spines, and primate lumbar and cervical spines [64,268]. Large animal models are useful to analyze the safety and efficacy of regenerative therapies under conditions more similar to the human spine with respect to IVD geometry, mechanical loading, and nutritional requirements. Care must be taken, however, to avoid solely choosing a model based on the above characteristics without considering the fibrosity of the NP, curvature of the spine, and presence of notochordal cells. Excessive spinal curvature can create large discrepancies in mechanical loading between the human spine. In one study the beagle cervical spine was chosen to investigate whole tissue-engineered replacement IVDs in vivo; however, the curvature and angle of cervical IVDs led to instability and migration of the implanted constructs [79]. Notochordal cells are the developmental progenitors of NP cells and disappear before skeletal maturity in humans; however, they are persistent in some species throughout adulthood [268]. The presence of notochordal cells will actively regenerate NP cells and other cell populations, which tends to keep the NP more fluid-like as seen in young human IVDs rather than fibrous as is common in aging human adults.

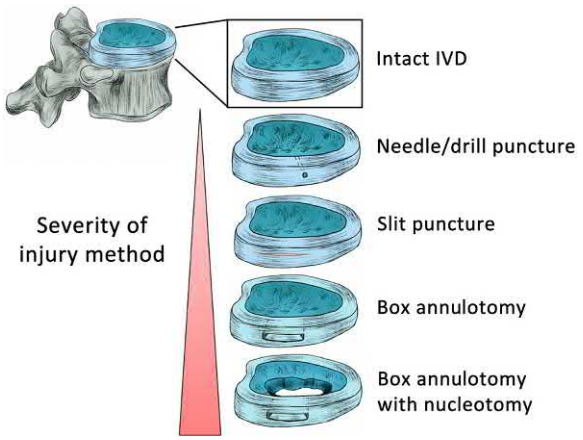

FIGURE 51.8 Representative injury methods to precipitate IVD degeneration for in vivo animal models.
A wide variety of injury methods have been used to initiate IVD degeneration in preclinical animal models. Needle and slit puncture are the most simple and consistent; however, they may not be aggressive enough to initiate degeneration. More aggressive injuries such as annulotomy and nucleotomy have a greater chance of precipitating degenerative changes to the IVD but are more difficult to employ and the amount of tissue removed is variable. *IVD*, Intervertebral disk. *Adapted from Sloan, et al. Tissue Eng, B 2018.*

As with the small animal models, there are a variety of methods to precipitate IVD degeneration if the selected animal model does not degenerate spontaneously. In large animal models, mechanical injury is the commonly used method, where injury/removal of the AF and NP leads to degeneration over time in vivo [63]. Groups have demonstrated successful IVD degeneration in vivo using slit/stab injuries, needle punctures, box annulotomies, and box annulotomies with nucleotomy [64]. The aggressiveness of the injury method dictates the progression of IVD degeneration, where small injuries lead to low levels of degeneration if any, while larger injuries can lead to herniation and collapse of the disk space (Fig. 51.8). Elliott et al. found that degeneration is proportional to the size of the injury relative to disk height and that on average injuries over 40% of the disk height have a high probability to initiate degeneration [63]. Besides mechanical injury, injection of chemolytic agents into the IVD such as chondroitinase ABC and chymopapain have been shown to

induce degeneration over time [265,269]. Varying degrees of degeneration can be achieved by tuning the concentration of the injected substances.

Concluding remarks

Efforts to regenerate and replace the tissues of the IVD have virtually exploded over the last two decades, although the field remains in its infancy. The complexity of the diverse degenerative and pathological processes that affect the IVD, as well as the intrinsic complexity of the heterogeneous disk structures, require that multiple strategies be developed for the treatment of the IVD. Development of strategies using cells, biologics, or gene therapy is often focused upon restoration of a single tissue source, such as NP or AF, and with or without biomaterial scaffolds. However, a growing number of tissue-engineering solutions have been proposed to integrate multiple tissues in the repair process, and additional work to promote integration amongst native, neo-generated, and implanted tissues will be critical to restoring IVD function. Many of the identified strategies derive largely from knowledge gained in cartilage tissue engineering, although the differing cellular, functional, and structural requirements of the IVD suggest that custom approaches are needed. Advances in IVD cell biology are needed to enable the identification of novel therapeutic targets, to select for classes of biomaterials, and to suggest appropriate drug delivery strategies, as disk cell phenotype, cell-biomaterial interactions, and the biology of aging for these cells, are still poorly understood. While a diverse array of molecules, cell sources, and materials are suggested as appropriate for IVD regeneration, additional work is needed to reveal some common and unique themes in human IVD cell responses that focus research on IVD specific strategies. Currently, underway clinical trials of autologous cell therapies or autologous protein products will pave the way for later generations of cellular and biologic-based therapies, as they are expected to illustrate the unique challenges of treating the pathologic and aged human IVD. The next decade promises great advances in the translation of basic and applied sciences to the clinical treatment of IVD regeneration and replacement.

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Chapter 52

Articular cartilage injury

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Introduction

In an effort to decrease the risk of posttraumatic osteoarthritis (OA), surgeons and scientists have been seeking ways to prevent progressive joint degeneration following injury. Surgeons have used operative treatments, including penetrating subchondral bone, soft-tissue grafts, and cell transplants, to stimulate restoration of damaged articular surfaces, with variable results. This chapter covers recent advances in efforts to prevent posttraumatic OA and in the use of artificial matrices, growth factors, and immature chondrocytes, or stem cells to promote cartilage repair as well as work suggesting that several biologic agents, including caspase inhibitors, antioxidants, and anti-inflammatory drugs, may minimize the effects of mechanical damage to chondrocytes.

Mechanical loading of articular surfaces in excess of the tolerances of those surfaces damages chondrocytes and their matrix; this damage can cause joint degeneration leading to the clinical syndrome of posttraumatic OA. The risk of posttraumatic OA depends on the type and severity of the injury and also on the repair and remodeling of the damaged articular surfaces. Three classes of joint injuries can be identified, based on the type of articular surface damage:

1. chondral damage and, in some cases, subchondral bone damage that does not cause visible disruption of the articular cartilage;
2. mechanical disruption of the joint surface limited to articular cartilage (chondral ruptures or tears); and
3. mechanical disruption of articular cartilage and subchondral bone (articular surface fractures).

In most instances, chondrocytes can repair damage that does not disrupt the articular surface if they are protected from further injury. Mechanical disruption of articular cartilage stimulates chondrocyte synthetic activity, but this response, with few if any exceptions, fails to repair the tissue damage. Disruption of articular cartilage and subchondral bone stimulates chondral and bone repair. The osteochondral repair response usually heals the bony injury, but the chondral repair tissue does not duplicate the properties of normal articular cartilage.

Normal pain-free movement depends on the unique properties of the articular cartilage that forms the bearing surfaces of synovial joints [1]. Degeneration of this remarkable tissue causes OA: joint pain and dysfunction that limit mobility [2,3]. The mechanisms, frequency, and natural history of articular surface injuries are poorly understood, but it is clear that these injuries can lead to posttraumatic OA [1,4–6]. Limited awareness of chondral and osteochondral injuries and difficulty in diagnosing many of these injuries makes it impossible to determine accurately their incidence, or their relationship to the development of joint degeneration [1,7,8]. However, arthroscopic examinations of injured knee joints suggest that closed articular surface injuries occur frequently [9,10]. One group of surgeons arthroscopically examined 85 knees with traumatic hemarthrosis but absent or negligible ligamentous instability [9]. Twenty percent of these knees had articular surface defects. In many patients cartilage injuries occur in association with injuries to other joint tissues, including menisci, ligaments, joint capsule, and synovium. In these people the cartilage injury may be overlooked; even when it is identified, it is difficult to distinguish the effects of the cartilage injury from the effects of the injuries to the other tissues. Damage to articular surfaces that does not result in visible disruption of articular cartilage or subchondral bone is not easily detected, although it probably occurs far more frequently than chondral and osteochondral fractures [4,6].

Recent advances in methods of diagnosing articular surface injuries, including arthroscopy and magnetic resonance imaging [11–13], combined with reports of new

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methods of stimulating cartilage repair or regeneration and osteochondral transplantation have increased interest in these injuries. Clinical evaluation of patients with articular surface damage and determination of the appropriate role of new treatments or the need for any treatment requires an understanding of the mechanisms of these injuries and their natural history. This chapter discusses the relationship between articular surface injury and joint degeneration, mechanisms of articular surface injuries, the responses of articular surfaces to injury and approaches to preventing joint degeneration following joint injury.

Articular cartilage injury and joint degeneration

The end stage of posttraumatic OA, the OA that follows joint injury, is identical to that of primary OA. In contrast to primary OA, which primarily affects older individuals and does not have a known inciting event, patients with posttraumatic OA are often young or middle-aged adults and have a well-defined precipitating insult [14,15]. Clinical experience and epidemiologic studies show that meniscal, ligament, and joint-capsule tears, joint dislocations, and intraarticular fractures increase the risk of the progressive joint degeneration and posttraumatic OA [14,16,17]. Participation in sports that expose joints to high levels of impact or torsional loading also increases the risk of joint degeneration [8,15].

The risk of OA following joint injury varies with the type of injury: meniscal and ligamentous injuries have a lower risk than intraarticular fractures. Gelber et al. [14] found that 13.9% of those who had a knee injury (including meniscal, ligamentous, or bone injuries) during adolescence or young adulthood developed knee OA, as compared with 6.0% of those who did not have a knee injury. A study of patients who suffered ligamentous and meniscal injuries of the knee reported that they had a tenfold increased risk of OA compared with patients who do not have joint injuries [18]. Intraarticular fractures have the greatest risk of OA. Depending on the severity of the injury and on the joint, the risk ranges from about 25% to more than 50% of patients [15,19].

The time interval between joint injury and the development of OA varies from less than a year in patients with severe intraarticular fractures to a decade or more in some patients with ligamentous or meniscal injuries [15,19]. Because many joint injuries occur in young adults, the population of patients with posttraumatic OA includes many individuals under 50 years of age. But older individuals may have an increased risk of OA after joint injuries. Studies of patients with intraarticular fractures of the knee show that patients older than 50 years of age have a two- to fourfold greater risk of developing OA than younger patients; patients over 40 years who have acetabular fractures and patients over 50 years who have displaced ankle fractures may also have a greater risk of OA than younger patients who have similar injuries; and age increases the risk of knee joint degeneration after anterior cruciate ligament (ACL) injury [15,20].

Mechanisms of articular cartilage injuries

Understanding the mechanisms of articular surface injuries requires appreciation of how loads and rate of loading affect articular cartilage [6,21]. Slowly applied loads and suddenly applied loads differ considerably in their effects. The articular cartilage extracellular matrix (ECM) consists of water and a macromolecular framework formed primarily by collagens and large aggregating proteoglycans [1]. The collagens give the tissue its form and tensile strength, and the interaction of aggregating proteoglycans with water gives the tissue its stiffness to compression, resilience, and probably its durability. Loading of articular surfaces causes movement of fluid within the articular cartilage matrix that dampens and distributes loads within the cartilage and to the subchondral bone [22]. When this occurs slowly, the fluid movement allows the cartilage to deform and decreases the force applied to the matrix macromolecular framework. When loading is too rapid for normal tissue deformation and fluid flow to occur through, as with sudden impact or torsional loading of the joint surface, the matrix macromolecular framework sustains a greater share of the force. If this force is great enough, it ruptures the matrix macromolecular framework, damages cells, and exceeds the ability of articular cartilage to prevent subchondral bone damage by dampening and distributing loads.

In vivo, expected and unexpected, slow and sudden articular surface loading may differ in the amount of force transmitted to joint surfaces. Muscle contractions absorb much of the energy and stabilize joints during slow, expected movements or impacts. Sudden or unexpected movements or impacts may occur too rapidly for muscle contractions to stabilize joints and decrease the forces on the articular surfaces. For this reason, sudden and unexpected movements or impacts can transmit greater contact stresses to joint surfaces.

Acute or repetitive blunt joint trauma can damage articular cartilage and the calcified cartilage zone—subchondral bone region while leaving the articular surface intact [4,23–26]. The intensity and type of joint loading that can cause chondral and subchondral damage without visible tissue disruption has not been well defined. Physiologic levels of joint loading do not appear to cause joint injury and are necessary to maintain articular cartilage, but impact loading above that associated with normal activities but less than that necessary to produce cartilage disruption can

cause alterations of the cartilage matrix and damage chondrocytes [4,23,24,26–28]. Experimental evidence shows that loss of proteoglycans or alteration of their organization (in particular, a decrease in proteoglycan aggregation) occurs before other signs of cartilage injury following impact loading. The loss of proteoglycans may be due either to increased degradation of the molecules or to decreased synthesis. Significant loss of matrix proteoglycans decreases cartilage stiffness and increases its permeability. These alterations may cause greater loading of the remaining macromolecular framework, including the collagen fibrils, increasing the vulnerability of the tissue to further damage from loading. These injuries may cause other matrix abnormalities besides loss of proteoglycans, such as distortions of the collagen fibril meshwork and disruptions of the collagen fibril proteoglycan relationships and swelling of the matrix, and they may injure chondrocytes [4,26].

Currently, there is no clinically applicable method of detecting alterations in articular cartilage that predictably lead to OA; however, new imaging techniques may provide methods of assessing articular cartilage composition and changes in cartilage thickness that inevitably lead to OA, including advances in magnetic resonance imaging, contrast enhanced μCT, and standing CT [29]. When probing the articular surface, surgeons sometimes find regions of apparent softening that may result from alterations in the matrix, and devices are being developed that will allow in vivo measurement of articular surface stiffness. Combined with information about cartilage composition, these measurements may make it possible to better define injuries to the articular surface that do not result in visible tissue disruption.

Disrupting a normal articular surface with a single impact requires substantial force, presumably because of the ability of articular cartilage and subchondral bone to dampen and distribute loads. A transarticular load of 2170 N applied to canine patellofemoral joints caused fractures in the zone of calcified cartilage visible by light microscopy and articular cartilage fissures that extended from the articular surface to the transitional or superficial radial zone of the articular cartilage [24]. A study of the response of human articular cartilage to blunt trauma showed that articular cartilage could withstand impact loads of up to 25 N/mm^2 (25 MPa) without apparent damage. Impact loads exceeding this level caused chondrocyte death and cartilage fissures [30]. The authors suggested that reaching a stress level that could cause cartilage damage required a force greater than that necessary to fracture the femur. Another study [31] measured the pressure on human patellofemoral articular cartilage during impact loading and found that impact loads less than the level necessary to fracture bone caused stresses greater than 25 MPa in some regions of the articular surface. With the knee flexed 90 degrees, 50% of the load necessary to cause a bone fracture produced joint pressures greater than 25 MPa for nearly 20% of the patellofemoral joint. At 70% of the bone fracture load, nearly 35% of the contact area of the patellofemoral joint pressures exceeded 25 MPa, and at 100% of the bone fracture load 60% of the patellofemoral joint pressures exceeded 25 MPa. These latter results show that impact loads can disrupt cartilage without fracturing bone.

Other experimental investigations show that repetitive impact loads can split articular cartilage matrix and initiate progressive cartilage degeneration [32–34]. Cyclic loading of human cartilage samples in vitro caused surface fibrillation [33]. Periodic impact loading of bovine metacarpal phalangeal joints in vitro combined with joint motion caused degeneration of articular cartilage [35]. Repeated overuse of rabbit joints in vivo combined with peak overloading caused articular cartilage damage including formation of chondrocyte clusters, fibrillation of the matrix, thickening of subchondral bone, and penetration of subchondral capillaries into the calcified zone of articular cartilage [34]. The extent of cartilage damage appeared to increase with longer periods of repetitive overloading, and deterioration of the cartilage continued following cessation of excessive loading. This latter finding suggests that some cartilage damage is not immediately visible.

An investigation of cartilage plugs also showed that repetitive loading disrupted the tissue and that the severity of the damage increased with increasing load and increasing number of loading cycles [36]. Two-hundred-and-fifty cycles of 1000 $lb/in.^2$ compression caused surface abrasions. Five-hundred cycles produced primary fissures penetrating calcified cartilage, and 1000 cycles produced secondary fissures extending from the primary fissures. After 8000 cycles, the fissures coalesced and undermined cartilage fragments. Higher loads caused similar changes with fewer cycles. The experiments suggested that repetitive loading can propagate vertical cartilage fissures from the joint surface to calcified cartilage and cause extension of oblique fissures into areas of intact cartilage.

Clinical studies have identified articular cartilage fissures, flaps, and free fragments and changes in subchondral bone similar to those produced experimentally by single and repetitive impact loads [4,37]. In at least some patients, acute impact loading of the articular surface or twisting movements of the joint apparently caused these injuries. In other patients, the cartilage damage may have resulted from repetitive loading. Magnetic resonance imaging of joints soon after an acute impact or torsional load occasionally shows changes in subchondral bone consistent with damage to the zone of calcified cartilage and subchondral bone, even when the articular surface is intact [11–13,38].

Clinical experience suggests that chondral ruptures or tears and osteochondral fractures result from similar impact and twisting-joint injuries, but they tend to occur in different age groups, and some individuals may have a greater risk of chondral tears. Chondral tears generally occur in skeletally mature people, while osteochondral fractures typically occur in skeletally immature people or young adults. This difference may result from age-related changes in the mechanical properties of the articular surface, including the uncalcified cartilage, the calcified cartilage zone, and the subchondral bone, that is, age-related alterations in the articular cartilage matrix decrease the tensile stiffness and strength of the superficial zone, and the calcified cartilage zone–subchondral bone region mineralizes fully following the completion of skeletal growth, presumably creating a marked difference in mechanical properties between the uncalcified cartilage and the calcified cartilage subchondral bone region. Taken together, these changes probably increase the risk of ruptures of the superficial-cartilage matrix and of these ruptures extending to the calcified cartilage subchondral bone region. Genetically determined abnormalities of the articular cartilage may also increase the risk of chondral ruptures from a given impact or torsional load, but the relationships between known genetic abnormalities of articular cartilage and cartilage properties have not been well defined.

Response of articular cartilage to injury

Articular surface injuries can be classified based on the type of tissue damage and the repair response:

1. cartilage matrix and cell injuries, that is, damage to the cartilage that does not cause visible mechanical disruption of the articular surface;
2. chondral fissures; flap tears, or chondral defects, that is, visible mechanical disruption of articular cartilage limited to articular cartilage; and
3. osteochondral injuries, that is, visible mechanical disruption of articular cartilage and bone (Table 52.1).

Matrix and cell injuries

Acute or repetitive blunt trauma, including excessive impact loading, can cause alterations in the articular cartilage matrix, including a decrease in proteoglycan concentration and possibly disruptions of the collagen fibril framework. Injuries that do not cause an apparent articular cartilage injury, including joint dislocations or ligament and joint capsule tears, may have associated damage to the articular cartilage cells and matrix [39]. The ability of chondrocytes to sense changes in matrix composition and to synthesize new molecules makes it possible for them to repair damage to the macromolecular framework [40]. It is not clear at what point this type of injury becomes irreversible and leads to progressive loss of

TABLE 52.1 Chondral and osteochondral injuries.

Injury	Clinical presentation	Repair response	Potential for healing
Damage to chondral matrix and/or cells and/or subchondral bone without visible disruption of the articular surface	No known symptoms, although subchondral-bone injury may cause painInspection of the articular surface and current clinical imaging methods for articular cartilage lesion may cause injuryImaging of subchondral bone may show abnormalities	Synthesis of new matrix macromolecules and cell proliferation	If the basic matrix structure remains intact and enough viable cells remain, the cells can restore the normal tissue compositionIf the matrix and/or cell population sustains significant damage or if the tissue sustains further cannot detect this type of damage, the lesion may progress to cartilage degeneration
Cartilage disruption (chondral fractures or ruptures)	May cause mechanical symptoms, synovitis, pain, and joint effusions	No fibrin clot formation or inflammation and synthesis of new matrix macromolecules and cell proliferation, but new tissue does not fill the cartilage defect	Depending on the location and size of the lesion and the structural integrity, stability, and alignment of the joint, the lesion may or may not progress to cartilage degeneration
Cartilage and bone disruption (osteochondral fractures)	May cause mechanical symptoms, synovitis, pain, and joint effusions	Formation of a fibrin clot, inflammation, invasion of new cells, and production of new chondral and osseous tissue	Depending on the location and size of the lesion and the structural integrity, stability, and alignment of the joint, the lesion may or may not progress

articular cartilage. Presumably, the chondrocytes can restore the matrix as long as the loss of matrix proteoglycan does not exceed what the cells can rapidly produce, if the fibrillar collagen meshwork remains intact, and if enough chondrocytes remain viable. When these conditions are not met, the cells cannot restore the matrix, the chondrocytes will be exposed to excessive loads, and the tissue will degenerate. Recent analyses of cartilage anabolic/catabolic turnover suggest that the collagen fibrils are largely not turned over during adulthood, whereas proteoglycan and other key structural molecules are continuously, sometimes rapidly turned over [41]. This description of the crucial role for production of macromolecules by chondrocytes during disease has led to increased focus on cell metabolic machinery. Recently investigators have found compelling evidence that chondrocyte mitochondrial dysfunction has a central role in the development of OA following joint injury and that preventing mitochondrial dysfunction can prevent or decrease the severity of post-joint injury OA [42].

Chondral injuries

Acute or repetitive trauma can cause focal mechanical disruption of articular cartilage, including fissures, chondral flaps or tears, and loss of a segment of articular cartilage [4]. The lack of blood vessels and lack of cells that can repair significant tissue defects limit the response of cartilage to injury [43,44]. Chondrocytes respond to tissue injury by proliferating and increasing the synthesis of matrix macromolecules near the injury. But the newly synthesized matrix and proliferating cells do not fill the tissue defect, and soon after injury the increased proliferative and synthetic activity ceases.

Osteochondral injuries

Unlike injuries limited to cartilage, injuries that fracture subchondral bone cause hemorrhage and fibrin clot formation and activate the inflammatory response [43–45]. Soon after injury, blood escaping from the damaged-bone blood vessels forms a hematoma that temporarily fills the injury site. Fibrin forms within the hematoma and platelets bind to fibrillar collagen. A continuous fibrin clot fills the bone defect and extends for a variable distance into the cartilage defect. Platelets within the clot release vasoactive mediators and growth factors or cytokines (small proteins that influence multiple cell functions, including migration, proliferation, differentiation, and matrix synthesis). These cytokines include transforming growth factor beta and platelet-derived growth factor. Bone matrix also contains growth factors, including transforming growth factor beta, bone morphogenic protein, platelet-derived growth factor, insulin-like growth factor I, insulin-like growth factor II, and others. Release of these growth factors may have an important role in the repair of osteochondral defects. In particular, they stimulate vascular invasion and migration of undifferentiated cells into the clot and influence the proliferative and synthetic activities of the cells. Shortly after entering the tissue defect, the undifferentiated mesenchymal cells proliferate and can begin to synthesize a new matrix. Within 2 weeks of injury, some mesenchymal cells assume the rounded form of chondrocytes and begin to synthesize a matrix that contains type II collagen and a relatively high concentration of proteoglycans. These cells produce regions of hyaline-like cartilage in the chondral and bone portions of the defect. Six to eight weeks after injury, the repair tissue within the chondral region of osteochondral defects contains many chondrocyte-like cells in a matrix consisting of type II collagen, proteoglycans, some type I collagen, and noncollagenous proteins. Unlike the cells in the chondral portion of the defect, the cells in the bony portion of the defect produce immature bone, fibrous tissue, and hyaline-like cartilage. Over time, this tissue remodels form normal bone.

The chondral repair tissue typically has a composition and structure intermediate between that of hyaline cartilage and fibrocartilage, and it rarely, if ever, replicates the elaborate structure of normal articular cartilage [1,46–48]. Occasionally, the cartilage repair tissue persists unchanged or progressively remodels to form a functional joint surface. But in most large osteochondral injuries, the chondral repair tissue begins to show evidence of depletion of matrix proteoglycans, fragmentation and fibrillation, increasing collagen content, and loss of cells with the appearance of chondrocytes within a year or less. The remaining cells often assume the appearance of fibroblasts as the surrounding matrix comes to consist primarily of densely packed collagen fibrils. This fibrous tissue usually fragments and often disintegrates, leaving areas of exposed bone. The inferior mechanical properties of chondral repair tissue may be responsible for its frequent deterioration [1,44]. Even repair tissue that successfully fills osteochondral defects is less stiff and more permeable than normal articular cartilage, and the orientation and organization of the collagen fibrils in even the most hyaline-like cartilage repair tissue do not follow the pattern seen in normal articular cartilage. In addition, the repair tissue cells may fail to establish the normal relationships between matrix macromolecules, in particular, the relationship between cartilage proteoglycans and the collagen fibril network. The decreased stiffness and increased permeability of repair cartilage matrix may increase loading of the macromolecular framework during joint use, resulting in progressive structural damage to the matrix collagen and proteoglycans, thereby exposing the repair chondrocytes to excessive loads and further compromising their ability to restore the matrix.

Clinical experience and experimental studies suggest that the success of chondral repair in osteochondral injuries may depend to some extent on the severity of the injury, as measured by the volume of tissue or surface area of cartilage injured and the age of the individual [49]. Smaller osteochondral defects that do not alter joint function heal more predictably than larger defects that may change the loading of the articular surface. Potential age-related differences in healing of chondral and osteochondral injuries have not been thoroughly investigated, but bone heals more rapidly in children than in adults, and the articular cartilage chondrocytes in skeletally immature animals show a better proliferative response to injury and synthesize larger proteoglycan molecules than those from mature animals [40,50–54]. Furthermore, a growing synovial joint has the potential to remodel the articular surface to decrease the mechanical abnormalities created by a chondral or osteochondral defect.

Preventing joint degeneration following injury

Orthopedic surgeons routinely perform extensive surgical procedures, some having substantial complication rates, in an effort to restore the alignment and congruity of articular surfaces following intraarticular fractures [19]. The purpose of these procedures is to decrease residual joint incongruity and thereby to decrease focal elevations of contact stress, presumed to be responsible for posttraumatic OA. These widely accepted practices are based largely on the assumption that joints are less likely to develop OA if the peak stresses on focal areas of the articular surface are reduced. However, there is little evidence to guide surgeons in determining how much stress the articular surface can tolerate, in the form of either acute impact or chronically increased stress, and the potential for human joints to repair and remodel the articular surface after injury is poorly understood.

The importance of mechanical forces in the pathogenesis of OA has led to the inception of new minimally invasive, nonsurgical manipulations of the biological responses to joint injuries with a high risk of posttraumatic OA aimed at preventing or delaying the disease. For example, the work of D'Lima et al. [55–57] shows that caspase inhibition can decrease mechanically induced chondrocyte apoptosis. Haut et al. have reported that P188 surfactant can limit chondrocyte necrosis following impact loading [58,59].

Other investigations indicate that antioxidants can prevent mechanically induced chondrocyte damage [42,57,58]. These studies showed that chondrocytes are subject to oxidative stress and damage induced by various mechanical insults to cartilage, including single impacts and chronic overloading [42]. Chondrocyte mitochondria respond to overloading by overproducing superoxide ($O_2^{\bullet -}$) from complex I of the electron transport chain in a tissue strain–dependent manner [42]. $O_2^{\bullet -}$ is a free radical that damages cellular proteins, lipids, and nucleic acids. It is rapidly dismuted to hydrogen peroxide (H_2O_2), which also causes oxidative damage to the cell. Excessive oxidant production can therefore lead to cell death, or irreversible disruption of mitochondrial mass or activity as well as permanent deficits in glycolysis in surviving cells, each of which undermines protein synthesis and ECM stability [42]. Recent reports indicate that interventions limiting $O_2^{\bullet -}$ production (amobarbital) or that aid in the detoxification of H_2O_2 by cellular reductases (*n*-acetylcysteine) block these harmful effects and delay the development of posttraumatic OA in a porcine intraarticular fracture model, underscoring the centrality of oxidative stress to cartilage injury [42].

Promoting articular surface repair

Better understanding of articular cartilage injuries and recognition of the limitations of the natural repair responses have contributed to the widespread interest in cartilage repair and regeneration [4,43,44,60–62]. In the last four decades, clinical and basic scientific investigations have shown that implantation of artificial matrices, growth factors, perichondrium, periosteum, and transplanted chondrocytes and mesenchymal stem cells can stimulate formation of cartilaginous tissue in synovial joint osteochondral or chondral defects [1,16,60,62–66]. In addition, chondrogenic progenitor cells residing in articular cartilage are chemotactically drawn to sites of cartilage injury and in the presence of chondrogenic growth factors regenerate mechanically competent hyaline cartilage. Use of these cells offers an exciting approach to research directed toward regeneration of articular cartilage using autologous cells.

Penetration of subchondral bone

Experimental and clinical investigations show that penetration of subchondral bone leads to formation of fibrocartilaginous repair tissue on the articular surfaces of synovial joints [16,44,59,64–66]. In regions with full-thickness loss or advanced degeneration of articular cartilage, penetration of the exposed subchondral bone disrupts subchondral blood vessels, leading to formation of a fibrin clot that fills the bone defect and usually covers the exposed bone surface [49,60]. If the surface is protected from excessive loading, undifferentiated mesenchymal cells migrate into the clot, proliferate, and differentiate into cells with the morphologic features of chondrocytes [67]. In most instances, over a period of six to eight weeks they form

bone in the osseous portion of the defect and fibrocartilaginous tissue in the chondral portion [43,68,69]. Initially, the chondral repair tissue can closely resemble articular cartilage in gross and light-microscopic appearance, but it fails to duplicate fully the composition, structure, and mechanical properties of normal articular cartilage, especially the types and concentrations of collagens and proteoglycans, and in many instances it will deteriorate with time [1,43,44,46].

Surgeons currently use a variety of methods of penetrating subchondral bone to stimulate formation of a new cartilaginous surface, including arthroscopic drilling and abrasion of the articular surface and making multiple small-diameter defects or fractures with an awl or a similar instrument, a method referred to as the microfracture technique [16,37,60,66,68,69]. Multiple authors report that these procedures can decrease the symptoms from isolated articular cartilage defects of the knee in a majority of patients [37,66,68–73].

Periosteal and perichondrial grafts

The potential benefits of periosteal and perichondrial grafts include introduction of a new cell population along with an organic matrix and some protection of the graft or host cells from excessive loading. Animal experiments and clinical experience show that perichondrial and periosteal grafts placed in articular cartilage defects can produce new cartilage [73,74]. O'Driscoll has described the use of periosteal grafts for the treatment of isolated chondral and osteochondral defects and demonstrated that these grafts can produce a new articular surface [16,64,75,76]. Other investigators have reported encouraging results with perichondrial grafts [77,78]. However, one study suggests that increasing patient age adversely affects the results of soft-tissue grafts. Seradge et al. [79] studied the results of rib perichondrial arthroplasties in 16 metacarpophalangeal joints and 20 proximal interphalangeal joints at a minimum of 3 years following surgery. Patient age was directly related to the results. One-hundred percent of the patients in their 20s and 75% of the patients in their 30s had good results following metacarpophalangeal joint arthroplasties. Seventy-five percent of the patients in their teens and 66% of the patients in their 20s had good results following proximal interphalangeal joint arthroplasties. None of the patients older than 40 years had a good result with either type of arthroplasty. The clinical observation that perichondrial grafts produced the best results in younger patients [79] agrees with the concept that age may adversely affect the ability of undifferentiated cells or chondrocytes to form an articular surface or that with age the population of cells that can form an articular surface declines [47,50,54].

Cell transplantation

Transplantation of chondrocytic cells grown in culture provides another method of introducing a new cell population into chondral and osteochondral defects. Experimental work has shown that both chondrocytes and undifferentiated mesenchymal cells placed in articular cartilage defects survive and produce a new cartilage matrix [1,80–84]. In addition to these animal experiments, orthopedic surgeons have used autologous chondrocyte transplants for treatment of localized cartilage defects [65,81–83,85,86]. Proponents of this procedure report that it produces satisfactory results, including the ability to return to demanding physical activities, in as many as 90% of patients [87,88].

Artificial matrices

Treatment of chondral defects with growth factors or cell transplants requires a method of delivering and in most instances at least temporarily stabilizing the growth factors or cells in the defect. For these reasons, the success of these approaches often depends on an artificial matrix. In addition, artificial matrices may allow, and in some instances stimulate, ingrowth of host cells, matrix formation, and binding of new cells and matrix to host tissue [89,90]. Investigators have found that implants formed from a variety of biological and nonbiological materials, including treated cartilage and bone matrices, collagens, hyaluronan, chitosan, fibrin, carbon fiber, hydroxyapatite, porous polylactic acid, polytetrafluoroethylene, polyester, and other synthetic polymers, facilitate restoration of an articular surface [1,74,90–92]. Lack of studies that directly compare different types of artificial matrices makes it difficult to evaluate their relative merits, but the available reports show that this approach may contribute to restoration of an articular surface.

Growth factors

Growth factors influence a variety of cell activities, including proliferation, migration, matrix synthesis, and differentiation. Many of these factors, including the fibroblast growth factors, insulin-like growth factors, and transforming growth factor betas, have been shown to affect chondrocyte metabolism and chondrogenesis [49,74,93,94]. Bone matrix contains a variety of these molecules, including transforming growth factor betas, insulin-like growth factors, bone morphogenic proteins, platelet-derived growth factors, and others [49,95]. In addition, mesenchymal cells, endothelial cells, and platelets produce many of these factors. Thus osteochondral injuries and exposure of bone due to loss of articular cartilage may release these agents, which affect the formation of cartilage repair tissue and have an important role

in the formation of new articular surfaces after currently used surgical procedures, including penetration of subchondral bone.

Local treatment of chondral or osteochondral defects with growth factors has the potential to stimulate restoration of an articular surface [94,96–98]. Despite the promise of this approach, the wide variety of growth factors, their multiple effects, the interactions among them, and the possibility that the responsiveness of cells to growth factors may decline with age [50,52–54] have made it difficult to develop a simple strategy for using these agents to treat articular surface injuries. However, development of growth factor–based treatments for isolated chondral and osteochondral injuries in combination with other approaches, including use of artificial matrices and cell transplants, appears promising [62,99].

Antiinflammatories

Posttraumatic synovitis is associated with joint injuries that cause OA [98]. Joint injury results in increases in proinflammatory cytokines in synovial fluids, which may promote cartilage degeneration. A clinical study indicated that anticytokine therapy relieved acute knee pain in patients with ACL tear [100], a result suggesting that such treatments could protect the joint from inflammatory damage. Why damage to joint tissues results in cytokine elevation and inflammation is unclear. However, results from studies of other organ systems show that inflammation is triggered by cell death resulting from tissue injury [99]. These observations strongly suggest that antioxidants, or drugs that downregulate immune responses to cell death, could block joint inflammation following a wide variety of joint injuries. Indeed, it was found that blocking innate immune responses to alarmins mitigated the development of posttraumatic synovitis in a mouse model of intraarticular fracture.

Conclusion

Articular surface injuries are a significant unsolved problem. They are common, the value of many current treatments is uncertain, and many of these injuries initiate progressive joint degeneration, a condition recognized as posttraumatic OA. Surgeons attempt to decrease the risk of posttraumatic OA by restoring joint stability, congruity, and alignment following injury. In addition, to promote repair and remodeling of damaged articular surfaces, they penetrate subchondral bone and insert periosteal and perichondral grafts and autologous chondrocytes. The results of these procedures vary considerably among patients, and there is limited information concerning the long-term outcomes. However, the available studies of people who have suffered joint injuries indicate that even with optimal current treatment the risk of posttraumatic OA is high following chondral tears and articular surface fractures. For this reason there is a clear need to improve the treatment of joint injuries. Clinical and experimental studies show that chondrocyte and mesenchymal stem cell transplantation, synthetic matrices, growth factors, and combinations of these treatments have the potential to restore articular surfaces. Other investigations suggest that biologic interventions may minimize chondrocyte damage due to mechanical forces. The most dramatic improvements in the treatment of articular surface injuries are likely to come from approaches that help maintain chondrocyte viability and function following joint injury, restore joint stability and congruity, and promote articular surface repair or regeneration and remodeling following joint injury.

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Chapter 53

Engineering cartilage and other structural tissues: principals of bone and cartilage reconstruction

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Introduction

Cartilage is a fiber-reinforced composite material composed of chondrocytes surrounded by specialized extracellular matrix (ECM) in unique architectures. There are three types of cartilage, namely, hyaline, elastic, and fibrocartilage. Hyaline cartilage is the most prevalent type of cartilage and can be found in synovial joints [1]. It is a translucent stratified tissue composed mainly of type II collagen that consists of several distinct microscale cellular niches [2]. Hyaline cartilage enables joints to articulate with low frictional forces by providing smooth and lubricated surface. One unique characteristic of hyaline cartilage is the lack of blood vessels, nerves, or lymphatics. It also has a limited capacity for intrinsic repair and healing. This type of cartilage is composed of a dense ECM with chondrocytes and the nutrients usually reach chondrocytes through diffusion. Hyaline cartilage chondrocyte homeostasis is achieved through cyclic compression, which facilitates the exchange of nutrients and waste products [3]. Elastic cartilage is similar to hyaline cartilage in architecture, and it contains yellow elastic fiber networks and type II collagen. The matrix is dominated by elastin fiber networks, and chondrocytes lie between the fibers [4]. Elastic cartilage is found in the ear, epiglottis, and pinnae. Fibrocartilage is composed of a mixture of types I and II collagen and is whitish in color. This type of cartilage is found mainly in soft tissue-to-bone attachments, symphysis pubis, annulus fibrosus, and menisci. Cartilage is largely populated by chondrocytes, which are specialized cells that can secrete proteins that form the ECM of cartilage. Collagen is the main constituent of cartilage and is made from amino acids wrapped together to form elongated triple-helix fibrils. Fibrils are structural materials that are usually in the scale of 10−100 nm [5]. Aggrecan, bone sialoproteins, and proteoglycans are also found in the ECM of cartilage.

Cartilage tissue has limited self-repair potential due to its avascular nature, and current surgical interventions are associated with mixed results. The concept of tissue-engineering (TE) approach to cartilage repair was first demonstrated in 1977 by a transplantation of chondrocytes into damaged cartilage [6]. Nowadays, autologous chondrocyte implantation (ACI) and matrix-induced ACI presented promising results [7,8]. However, these techniques are associated with complications such as graft failure, periosteal hypertrophy, and delamination. The most important factors to consider in regeneration of cartilage tissue are the underlying biology. The structure and composition of cartilage tissues are depth dependent, which are divided into superficial, middle, deep, and transitional zones depending on the alignment of collagen fibers and composition of proteoglycan. Despite their single cell type, chondrocytes present significant differences in morphology, organization, and density across the depth of cartilage. These topographical differences over the range of a few micrometers in relation to cartilage thickness and matrix contents exist [9−11].

Biomaterials for cartilage tissue engineering

The selection of biomaterial is critical to the success of TE approaches in cartilage repair. Biomaterials for cartilage

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repair can be selected from either natural or synthetic materials. Natural materials include various types of collagen-based materials, chitosan, hyaluronic acid (HA), gelatin, keratin, and fibrin hydrogels [12−15]. As for synthetic materials, mostly biodegradable polymers such as poly-glycolic acid (PGA), poly-L-lactic acid, or their composite copolymers poly lactic-*co*-glycolic acid (PLGA), also nonbiodegradable polymers such as polytetrafluoroethylene and polyethylmethacrylate have been studied [16,17]. Among them, collagen has been used widely to construct cartilage scaffolds because it is the most abundant protein in mammals. For instance, Funayama et al. developed chondrocyte-laden type II collagen scaffold and injected the scaffold into damaged rabbit cartilage without a periosteal graft [18]. After 8 weeks of the injection a favorable regeneration of hyaline cartilage was observed. Furthermore, a significant difference on cartilage regeneration was observed between transplanted and control groups at 24 weeks after the injection [18].

Besides chemical composition, material topography and architecture are critical for engineering cartilage tissue. For engineering cartilage scaffolds, various fabrication methods have been reported. It includes solid free fabrication, which allows the production of an anatomical meniscal scaffold using a fiber-deposited method with an XYZ robotic arm. The scaffolds are prepared from poly(ethylene oxide-terephthalate)-*co*-poly(butylene terephthalate) and analyzed using computerized tomography (CT) and magnetic resonance imaging (MRI) dataset [19]. Electrospinning technology has used to create aligned collagen fibers across the depth of cartilage [20]. A five-layer scaffold consisted of type I and II collagen fibers was fabricated with random and aligned orientations using electrospinning technology. After 7 days of static culture under growth factor, seeded MSCs within the electrospun fibers produced higher aggrecan and collagen II, which demonstrates chondrogenic differentiation of MSCs within the aligned scaffold [20]. As far as hyaline cartilage is concerned, few groups have tried to reconstruct the zonal organization mimicking the structure of native cartilage [9,10,21,22]. One approach would include seeding chondrocytes from specific zones in particular layers to reconstruct the multizonal topography of native cartilage [21,22]. This is supported by the finding that chondrocytes from different zones respond differently in terms of gene expression and matrix production. On the other hand, layering- or gradient-customized hydrogel could allow construct with zonal properties [10,22].

Cell sources for cartilage tissue engineering

There are multiple cell sources for the development of regenerative therapies for cartilage constructs. Adult chondrocytes or chondrocyte progenitor cells sourced from human tissues are an ideal logical way [23]. Mature differentiated chondrocyte isolation from adult tissues is possible, and at present, the technology is commercially available (e.g., Carticel and ChondroCelect). However, there are still drawbacks such as donor site morbidity, isolated cells present phenotypic instability in culture, and comparatively low yield of cells isolated from autologous tissue [24]. To overcome these drawbacks, research has focused on cells with potential to differentiate to a chondrogenic phenotype. Wang et al. used genetically modified cells such as dermal fibroblasts that were programed to differentiate into osteochondrogenic cells [25]. Some more attractive approaches have developed such as direct differentiation of human embryonic stem cells into chondrocytes [26] as well as chondrogenically differentiated induced pluripotent stem cells for cartilage defect repair [27]. However, safety concerns and differentiation protocols still need to be addressed.

MSCs for cartilage regeneration have been isolated from bone marrow, cartilage, periosteum, adipose tissue, synovial lining of large joints, muscle, and from embryonic tissues [23,28,29]. MSCs harvested from various tissues have been shown to have different potentials for chondrogenic differentiation. Among them, MSCs isolated from adipose tissues demonstrated an excellent potential as adipose tissue is in abundance in the human body, although some studies reported that adipose tissue-derived stem cells have poor chondrogenic potential [30]. Other challenges include seeding density, number of passages, biomaterials, and the use of growth factors to induce the chondrogenic phenotype. Chondrogenesis can be influenced by the concentration of chondrocytes in the construct. Li et al. showed that a concentration of 5.0×10^6 cells/mL can induce a higher chondrogenic output in comparison to lower and higher concentrations [31]. The passage number of the cells is another factor that can diminish the MSC to chondrocyte transition with studies suggesting that MSCs after passage 10 lose their chondrogenic differentiation [32]. No consensus in regard to the ideal scaffold for cartilage regeneration exists with the most common biomaterials to include PLGA, fibrin glue, hydrogel, collagen, tissue membrane, PGA, and alginate. Additives to the carrier have been used, including HA, culture media, plasma, and collagen [33−35]. Finally, growth factors to induce chondrogenic phenotype are required with transforming growth factor (TGF)-β1 or TGF-β3 and CDMP-1 and fibroblastic growth factor (FGF)-2 been the most commonly used [36]. It must be emphasized that, to date, there is no single generally accepted method, and the aforementioned techniques are still controversial.

Biofabrication of cartilage tissue

Magnetic resonance imaging and computerized tomography scans

Biofabrication of cartilage tissue requires a detailed knowledge of underlying anatomy. Three-dimensional (3D) graphics of cartilage can be generated through anatomical specimens by MRI scans, which later can be used in 3D printing of the scaffolds. Fig. 53.1 shows 3D graphics of cartilage generated from laser-scanning technology. High-resolution MRI is an emerging technology that is gradually being introduced into the clinical practice, and it enhances the resolution and sensitivity rate considerably [39,40]. X-ray and CT scans produce low value information as they do not capture cartilage, although they can show bone anatomy. It is essential to quantify both the thickness and the volume of the cartilage as it varies between anatomical areas. Substantial progress has been recently achieved in enhancing the imaging of cartilage physiology and detecting changes in proteoglycan content and collagen ultrastructure [37]. Another way to obtain a 3D model of the cartilage is by producing a 3D coordinate frame. Coordinate measuring machines can be used where a probe meets the sample, and the 3D coordinates are recorded. This technology has the capacity to capture the surface anatomy with a resolution of 1 μm. However, it cannot distinguish between tissues or provide information of the composition of multilayered tissues.

Scaffolds for cartilage tissue engineering

Scaffold-free fabrication of cartilage allows cartilage tissues to be grown in the lab and subsequently implanted to the area that needs to be treated [41]. Here, silicone molds are used to form petri dish, upon which the chondrocytes could grow. The agarose solution is poured on the silicone molds, which results in the micro-molded nonadhesive agarose hydrogel. The cell suspension is carefully implanted into spherical chambers at the bottom. After 18 hours, the cell suspensions coalesce and turn into spheroids that are then implanted into the body.

Hybrid scaffolds can be printed using digital light processing (DLP), a new water-based 3D printing method using photosensitive hybrid polymers such as polyurethane with HA [42]. The hybrid materials have high printing resolution and have shown nontoxic properties toward attached cells. In addition, 3D printed constructs promote good cell adhesion and could be customized for cartilage tissue reconstruction. Fig. 53.2 explains the fabrication of the cartilage tissue and its clinical application for cartilage repair. The key factor in 3D printing by using a DLP printer is the viscosity of the material, which affects the printing resolution and accuracy. The mixture of the resin is stirred at high speed while the material is heated to remove water. Photo-initiators and poly(2-hydroxyethylmethacrylate) are added to aid in the light curing, which results in a customizable print where the shape has an error of only 4% varying from the original design (Fig. 53.2A). Moreover, this customized hybrid scaffold shows high cytocompatibility with excellent

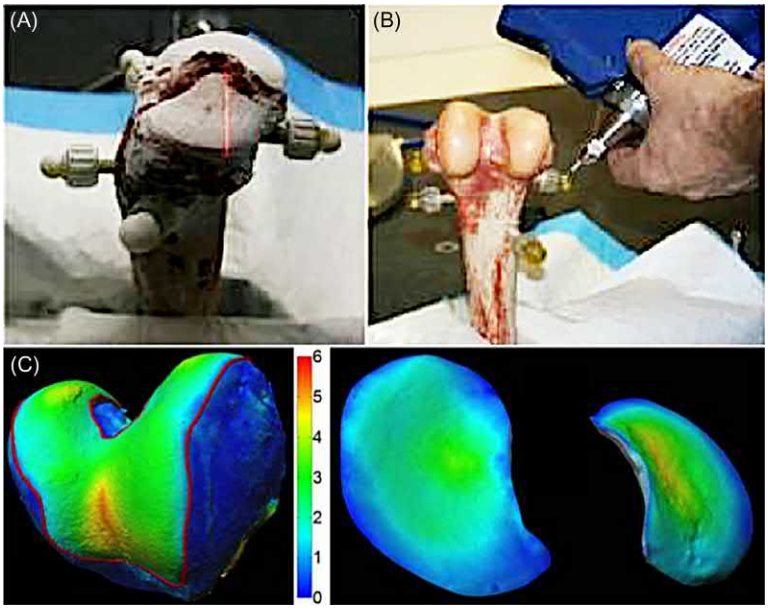

FIGURE 53.1 (A) Laser scanning and (B) physical marker probing of knee specimen [40]. (C) Laser scanner image of cartilage [41].

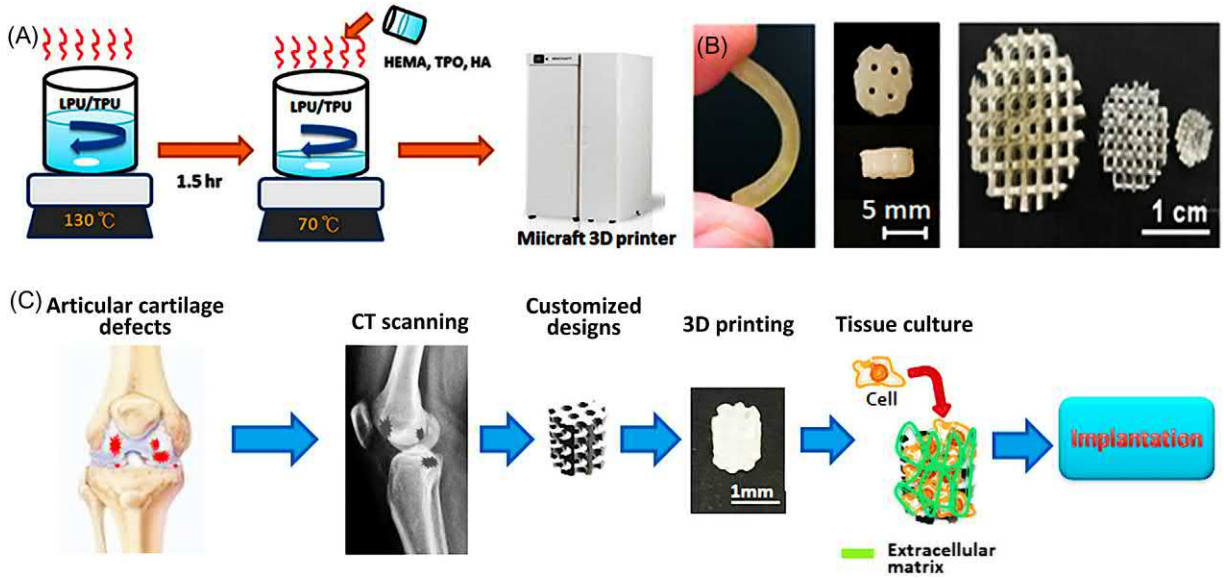

FIGURE 53.2 (A) The schematics of fabrication process of water-based PU with HA. (B) The images of the printed hybrid scaffolds. (C) Overall process of fabricating cartilage tissue and its clinical application. The CT images of the articular cartilage defects are applied to enable the design of 3D reconstruction scaffolds printed with PU/HA hybrid materials by DLP printing technology. Targeted cells are cultured into PU/HA scaffolds and the cartilage tissue can be used for implantation after in vitro tissue maturation. *3D*, Three-dimensional; *CT*, computerized tomography; *DLP*, digital light processing; *HA*, hyaluronic acid; *PU*, polyurethane. *Adapted from Shie MY, et al. 3D printing of cytocompatible water-based light-cured polyurethane with hyaluronic acid for cartilage tissue engineering applications. Materials (Basel) 2017;10(2):136.*

chondrogenic differentiation capacity and closely mimics the mechanical properties of articular cartilages [42].

Bioprinting techniques for fabrication of cartilage constructs

A number of studies have investigated the application of inkjet and micro-extrusion printing techniques using hydrogels for cartilage tissue engineering (TE). Micro-extrusion is a commonly used technique where bioinks are expelled while carrying a payload of feasible cells, genetic materials, or factors of growth [43]. Fig. 53.3 shows a schematic drawing for micro-engineered GelMA hydrogels production using several techniques at microscale.

In an early study, chondrocytes were extruded into basic geometries utilizing dextran-based hydrogel and HA [45]. Researchers observed high cell feasibility for up to 3 days in vitro showing the guarantee of such methods. In another study, chondrocytes were encapsulated in GelMA, and they were then extruded into simple porous structures for engineering grids [46]. Cells were alive for about 4 months in vitro and the researchers have shown collagen type II and sGAG generation inside the constructs. Another method for 3D bioprinting has additionally been investigated for engineering cartilage by employing droplet-producing bioprinting. Here, a discrete volume of bioink is deposited in a translating pattern rather than continuous strands as in micro-extrusion. These droplets can be produced by using either inkjet [47], acoustic droplet [48], or micro-valve [49] based technologies.

Bioinks for cartilage tissue printing

Hydrogels have been selected as attractive candidates for bioinks due to their highly tunable properties, cytocompatibility, and high water-content property. Table 53.1 provides a summary of different bioinks that are used for keeping cartilage fabrication up-to-date. Several types of bioinks were manifested to be theoretically sufficient for inkjet- and micro-extrusion-based bioprinting. These bioinks include GelMA [50], fibrin [51], collagen [52], various PEG-derivative hydrogels [53], agarose [54], and HA-based hydrogels [55]. These hydrogels represent excellent rheological properties, so it can be controlled easily during the micro-extrusion process. In addition, they provide structural similarity to ECM due to their high water-content property, which supports cellular viability and proliferation postprinting. Important parameters that must be considered during the design of bioinks are viscosity, shear thinning behavior, and yield stress. For instance, high cross-linking densities of hydrogel polymers are preferable because it possess high viscosity and yield stress, which enhances mechanical stability for extrusion process. In contrast, encapsulated cells take advantage of lower cross-linking densities of hydrogels

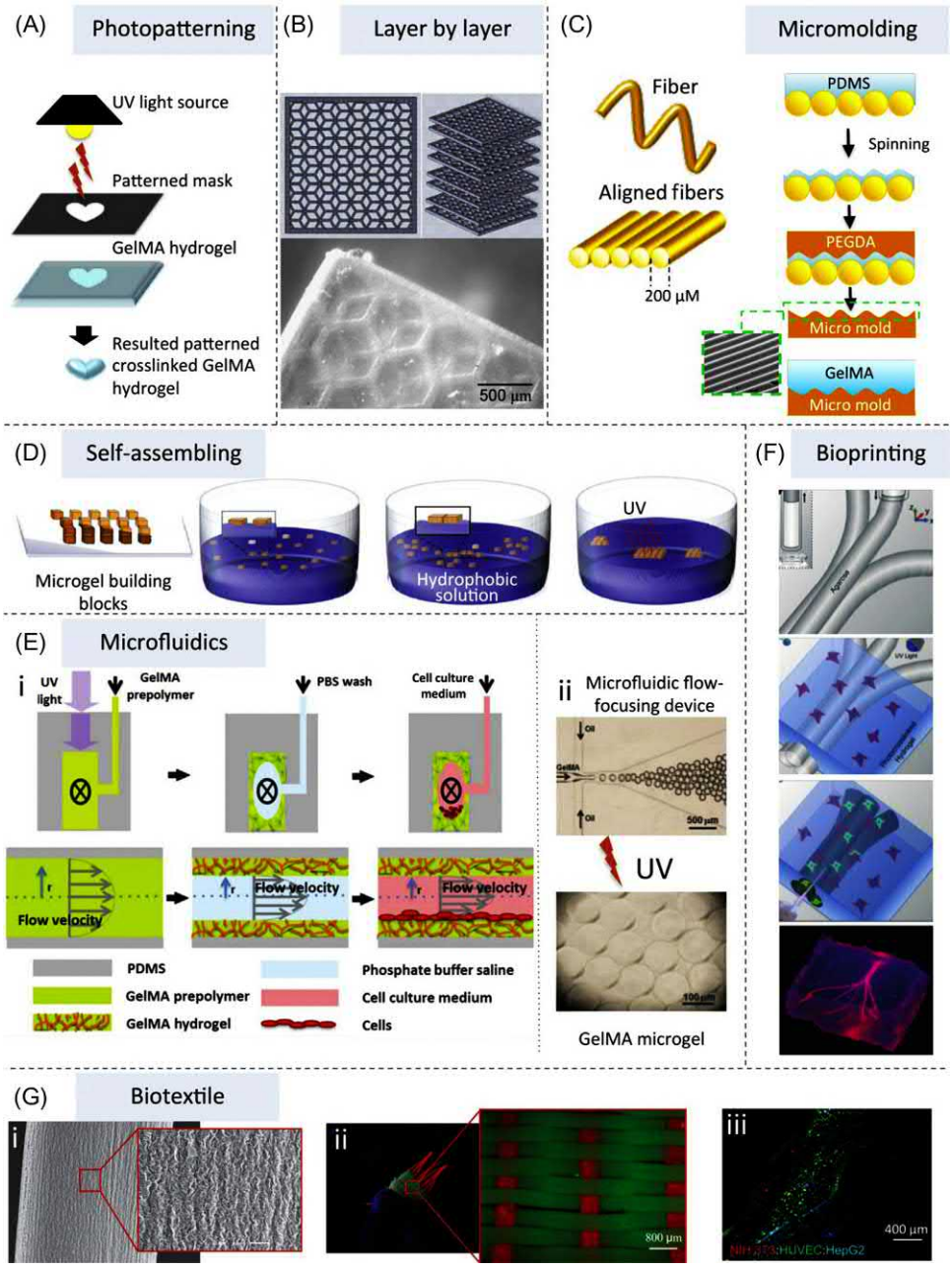

FIGURE 53.3 Microfabrication techniques used to produce GelMA hydrogels constructs: (A) photopatterning of GelMA using a prepatterned photomask; (B) stacked layers of patterned GelMA hydrogels fabricated using a micromirror projection stereolithography system; (C) fiber-assisted micromolding technique for the production of parallel microgrooved surfaces that serve as a template for micropatterning GelMA; (D) self-assembly of microgels fabricated by photo patterning; (E) examples of the microfluidics of GelMA hydrogels microfabrication: (i) coating microchannels with GelMA hydrogel and (ii) fabrication of spherical GelMA microhydrogels using a microfluidic flow-focusing device; (F) schematic representation of the bioprinting method for fabricating microchannels inside a GelMA hydrogel using an agarose template; (G) biotextile techniques as applied to the microfabrication of hybrid alginate and GelMA fibers and their assembly: (i) SEM image of fabricated fiber, (ii) typical woven fabric, and (iii) a braided construct from NIH 3T3 fibroblasts, HUVECs, and HepG2 as a liver model. *Adapted from Yue K, et al. Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. Biomaterials 2015;73:254–71 [44].*

for their proliferation and differentiation into targeted tissue [56]. The contradiction between these two parameters is determined as bioprinting window [56]. It is worth mentioning that small-sized nozzles with high extrusion pressure could lead high cell death rate postprinting due to the increased shear forces imposed on the cells. It was reported that less than 35% of cell viability was observed after extrusion at the shear stress of 60 kPa or higher [57]. Controlling the needle geometry can decrease shear forces encountered by the cells during the extrusion process. For example, cell viability could be increased at lower inlet pressure by using conical needles rather than cylindrical ones [58].

An early study demonstrated printing of layer-by-layer construct for cartilage repair using human articular chondrocytes encapsulated in a poly(ethylene glycol) dimethacrylate bioink. This study demonstrated the capability to fabricate cartilage matrix components using continuous inkjet printing of chondrocytes while maintaining cell viability and biological functionality within 3D printed scaffold. In another study, the same authors tested the way it was viable to leverage the method to an immediate print

TABLE 53.1 Bioinks for bioprinting of cartilage tissue.

Bioink	Chondrogenic capacity	Inherent printability	Resolution (μm)
Agarose	1	1 (Difficult to print high aspect ratios discarding the need for supporting structures)	30–100 [27]
Alginate	1	2 (Needs smart cross-linking methodologies/thickening agents to print constructs with higher fidelity)	1000 [27]
Sulfated Alginate	2 (Can bind growth factors, e.g., TGF and FGF and uses encapsulated bovine chondrocytes to induce for collagen II deposition and potent proliferation)	2 (Needs smart cross-linking methodologies/thickening agents to print constructs with higher fidelity)	650 [27]
Alginate/ nanocellulose	1 (The effect of the adding nanocellulose is still not studied)	3 (Nanocellulose imparts and a behavior of shear thinning)	400–600 [27]
GelMA	1	2 (Needs smart cross-linking methodologies/thickening agents to print constructs with higher fidelity)	50 [2]
HA	2	2	10–100 [28]
GelMA + (HA)	2 (Adding of HA improves chondrogenesis)	2 (Adding HA enhances rheological properties)	
GelMA/ gellan gum	1 (The addition of gellan gum does not improve chondrogenesis in comparison to GelMA alone)	3 (Adding gellan gum imparts a shear thinning behavior)	
PEGDMA	1	2 (Needs smart cross-linking methodologies/thickening agents to print constructs with higher fidelity)	
Collagen	1	2 (Needs high collagen with high densities for a controlled extrusion ~15 mg/mL)	100 [27]
Fibrin	1	1 (Needs smart cross-linking methodologies/thickening agents to print constructs with higher fidelity)	Can be improved to 1 with DLP printing [29]

Chondrogenic capacity = 1, indicates for a material that has unknown inherent chondro-inductive factors. Chondrogenic capacity = 2, indicates for a material that can support chondrogenesis of MSCs, excluding the need for the addition of growth factors such as TGF-β3. Inherent printability = 1, indicates for a material that cannot be easily for more than one layer reliably. Inherent printability = 2, indicates for a material where extrusion can relatively form simple shapes. Inherent printability = 3, indicates for a material where extrusion can relatively form complex shapes. *DLP*, Digital light processing; *FGF*, fibroblast growth factor; *HA*, hyaluronic acid; *PEGDMA*, poly(ethylene glycol) dimethacrylate; *TGF*, transforming growth factor.

right into a cartilage defect [59]. Matrix formation was assessed after 6 weeks regardless of a visible interface between the local and repaired cartilages, and the interface failure stress had significantly increased demonstrating the potential of this approach [60].

Combining alginate with cartilage matrix components such as chondroitin sulfate and HA can produce hydrogels with more biomimetic capabilities that support neocartilage formation [61]. In another study, it was found that it is possible to support cartilage tissue construction by using chondrocytes along with hMSCs [46]. In general, GeIMA has been used to support the development of fibrocartilage with a mixture of higher amount of type I collagen [62]. An addition of chondroitin sulfate and HA into GeIMA/collagen cartilage matrix enhanced the chondrogenesis. Moreover, the addition of these two cartilage matrix components resulted in a higher bioink viscosity, which improved printability significantly [45]. Similarly, a synthetic hydrogel having thermosensitive characteristics and constituted from a base triblock methacrylated polyHPMA-lac-PEG copolymer has been chemically combined with either methacrylated HA or methacrylated chondroitin sulfate for cartilage bioprinting. Another type of bioink comprising nanofibrillated cellulose and alginate was developed for cartilage engineering as a novel shear thinning bioink [63]. This type of shear thinning property has both high printability and high cell proliferation rate. The chondrogenic capabilities of these bioinks can be further improved by sulfated alginate components. Sulfated alginate can activate FGF

signaling that assists in maintaining chondrogenic phenotype. However, nanocellulose component in bioinks also performs as a thickening agent that induces shear stress through extrusion printing nozzles due to its high viscosity, which decreases chondrocyte proliferation and capability to synthesize ECM components [64].

Osteochondral tissue engineering

Osteochondral injury can happen at any joint; however, it is most common in the knee and ankle joints [65]. Such injuries can be well localized or can be the outcome of a complex injury associated with a fracture. Cartilage becomes torn or damaged and the progression of cartilage damage is closely associated with the extent of injury and the condition of the subchondral bone [32]. A genetic predisposition in terms of progression has been also hypothesized [66]. Osteochondral defects in the knee may occur in either femoral or tibial side [67]. In the ankle, such defects are frequently seen in the talus. Osteochondral defects cause pain and disability especially following prolonged use of the affected limb. Such injuries can be devastating for young people who are prone to these injuries through participation in athletic activities and contact sports.

The clinical management of osteochondral defects is challenging. Debridement of the loose and unstable osteochondral fragments might improve patient's symptoms. Microfracture is a procedure performed allowing the coverage of the defect by fibrocartilage. Alternatively, the osteochondral autograft transplantation surgery can be performed by harvesting and inserting osteochondral materials from nonweight-bearing areas to treat osteochondral defects [68]. Alternatively, ACI is designed to regenerate cartilage tissues with structural and functional features [69]. However, these techniques still have some drawbacks related to the donor site morbidity and graft failure due to peripheral chondrocyte death. Furthermore, there are several challenges that face the success of treatment of osteochondral defects. These include issues related to grafting, implant availability and biomechanical integrity, short cell viability, and risk of disease transmission for allografts. In addition, cell leaking in the articular space and loss of chondrogenic phenotype in the case of the ACI first-generation technique utilizing expanded chondrocytes has been voted as well as the need for two interventions delaying times and increasing costs for scaffold-based ACI [69,70].

3D bioprinted osteochondral constructs may provide a promising solution for treating arthritis associated with bony defects. Even in cases of isolated cartilage damage, treating the defect might not be sufficient as there is evidence to suggest that osteoarthritis is a disease affecting not only the cartilage but also the subchondral bone [32]. Hence, a holistic approach to treat arthritis is needed. In order to use this technology in a clinical trial, major steps have to be taken. The International Cartilage Repair Society is serving a leading role in analyzing the current state of scientific developments for the treatment of osteochondral lesions in order to provide recommendations for the execution of preclinical and clinical studies [71].

So far, there are several attempts to treat osteochondral defects. A number of researchers focused on the production of osteochondral constructs rather than cartilage patches [72–76]. Bone constructs loaded with chondrocytes showed the integration of cartilage on bone [73]. The authors concluded that such constructs with designed mechanical properties might offer alternative reconstruction procedures for the treatment of articular surfaces [73]. In one study, hyaline cartilage was created in a rabbit proximal humeral joint following bioprinting of Hydroxyapatite (HAp) powder and Polycaprolactone (PCL) [74]. The scaffold was infused with TGF-β3. By using this technique, promising results were also reported in cases of reconstruction of femoral head [76] and temporomandibular defect repair [75]. In summary, much progress has been made in the area of bone and cartilage TE. New technologies are being investigated to improve the quality and outcomes in this important area.

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Chapter 54

Tendon and ligament tissue engineering

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Introduction

Tendons and ligaments are collagenous soft tissues within the musculoskeletal system that serve important mechanical roles in providing joint stability and enabling mobility. The primary distinguishing feature between these connective tissues is what they connect: tendons transfer muscle forces to bones while ligaments form links between distinct bones. Due to the high mechanical demands placed upon these tissues, tendon and ligament are frequently injured, causing pain and disability to patients, while also representing a significant clinical burden [1]. Minor to moderate tendon and ligament injuries commonly occur in the form of subfailure strains, sprains, or repetitive use aggravation [2]. In addition, injuries that fully compromise the mechanical integrity of tendon and ligament are also common in the form of acute traumatic tears [e.g., anterior cruciate ligament (ACL), Achilles tendon], acute lacerations (e.g., flexor and extensor tendons), and/or chronic overuse damage (e.g., rotator cuff tendons, ulnar collateral ligament). Tendon and ligament injuries afflict people of all ages, with some tissues more commonly injured in young adults (e.g., ACL tear) and other injuries more often appearing in elderly (e.g., rotator cuff degeneration and tear) [3]. Some injuries track more closely based on patient occupation and/or hobbies, such as manual labor (e.g., flexor tendon laceration) or baseball pitching (e.g., elbow ligament fatigue damage). Although significant differences separate these various conditions, common features for most tendon and ligament injuries include pain, reduced activity, and disability from limited use of affected joints [4].

In general, the inherent healing response and regenerative capacity of tendon and ligament are quite low, such that recapitulation of functional tissue integrity typically requires clinical intervention [1]. Even then, successful treatment of many tendon and ligament injuries remains elusive. Nonsurgical treatments can sometimes provide pain relief and may improve motion, but such approaches are not useful in restoring proper function of torn tissues [5]. In that case, surgical repair can be performed by directly reapproximating torn tissue ends [6], reinforcing/augmenting a tissue repair using biomaterials [7], or fully replacing the damaged tendon and ligament with a biologic or synthetic graft [8]. In certain conditions, surgical reconstruction of damaged tissues can yield relatively successful clinical outcomes, but many procedures suffer from serious challenges and limitations. For example, while reconstructions performed using auto- and allografts may offer the best match for tissue properties, these procedures are impacted by donor site morbidity and limited supply of graft tissues, respectively [9]. Synthetic materials overcome these challenges but are not generally able to match the complex mechanical properties of tendon and ligament (e.g., anisotropic, inhomogeneous, nonlinear, viscoelastic), thereby limiting their utility, and are not capable of adapting to varying functional demands since they are not biologically active [8]. In addition, these graft-based repairs struggle to recapitulate proper interfaces between dissimilar tissue types (e.g., muscle−tendon, ligament−bone), which can lead to additional postsurgical complications [10]. Tissue engineering offers a very appealing solution for tendon and

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ligament repair and reconstruction due to the potential for such an approach to overcome many of the weaknesses and limitations of current strategies. For example, tissue engineering offers the potential for available supply, bioadaptability, interfacial ingrowth, and even patient-specific customization. This chapter will highlight some of the important considerations and ongoing challenges for tissue engineering of tendon and ligament.

Tendon and ligament composition, structure, and function

Composition

In general terms, tendon and ligament are composed of similar constituents that include collagen, proteoglycans, elastic fibers, glycoproteins, other minor proteins, and water [1]. While water makes up a large overall percentage of these highly hydrated tissues (~65%–75% wet weight), type I collagen comprises ~70%–80% of the dry weight and represents the primary structural component of tendon and ligament, providing significant mechanical strength when these tissues are subjected to tensile forces [11,12]. At different points in space and time, enzymatic and nonenzymatic cross-links are known to form between collagen molecules, the quantity and distribution of which affect the overall mechanical properties of the collagen network [13]. Various proteoglycans—with their corresponding negatively charged, water-binding glycosaminoglycan side-chains—are found within the tendon and ligament, including small leucine-rich proteoglycans such as decorin and biglycan, as well as larger proteoglycans such as aggrecan and versican. The amount and location of these various proteoglycans is believed to be predominantly due to differences in functional demands, where tissue regions subjected to compression typically exhibit higher quantities of proteoglycans to support these nontensile loads [14]. Other constituents such as glycoproteins and minor collagens may also be distributed differentially depending on requirements inherent to specific physiological loading environments [15]. Recent work has begun to describe a potentially important mechanical role for elastic fibers in tendon and ligament, which may contribute to the low-force response to load and/or energy-return for certain tendon and ligament with high demands (e.g., Achilles tendon); more work will be required to fully elucidate the role of elastic fibers and other minor tissue constituents of the extracellular matrix (ECM) [16]. In addition to these ECM components, tendon and ligament contain cells, most commonly fibroblasts but also progenitor cells, chondrocyte-like cells in some regions, and inflammatory cells in tendinopathic conditions [1]. While cells provide the bioresponsiveness and adaptability of tendon and ligament, the relatively low density of cells may contribute to the limited regenerative capacity of these tissues.

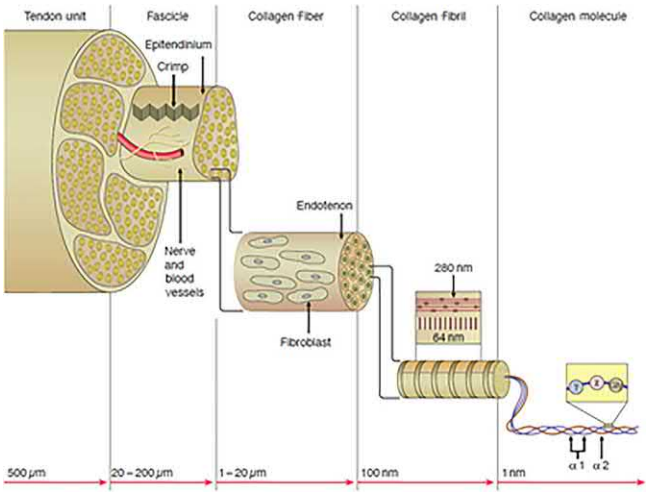

FIGURE 54.1 Hierarchical structure of tendons, where the organizational structure of collagen aggregates with increasing length scales. Ligaments exhibit a similar structural hierarchy. *Reprinted with permission from Zeugolis DI, Chan JCY, Pandit A. Tendons: engineering of functional tissues. In: Pallua N, Suscheck CV, editors. Tissue engineering: from lab to clinic. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011. p. 537–72 [17].*

Structure

Tendon and ligament are hierarchical structures with an organization that builds from the nanoscale to the centimeter scale, based primarily on the built-up form of type I collagen (Fig. 54.1). Starting with procollagen that weaves together to form collagen molecules, which are then packaged into fibrils, and subsequently fibers, fascicles, and whole tendon, the hierarchical structure of tendon and ligament is key to providing mechanical support necessary for proper function. For example, the degree to which collagen fibers are organized (e.g., uniformly aligned vs partially dispersed) in the direction of loading correlates with mechanical properties [18,19]. Other structural features are believed to contribute to important aspects of tendon and ligament function (e.g., collagen crimp at the fibril level likely contributes to the nonlinear stress–strain response). Also, various noncollagenous compositional constituents, such as proteoglycans and elastic fibers, may have roles at specific levels of the hierarchical structure of tendon and ligament. Taken together, the structural organization of tendon and ligament is fundamental to the primary purpose of supporting and transferring mechanical load.

Function

Although nearly all tendon and ligament share a common function that is primarily mechanical, there is significant

variability in the nature of the physiologic demands imposed upon each specific tissue throughout the body [2]. Specifically, the magnitude, frequency, duration, and direction of forces that connective tissues experience can vary dramatically in different anatomical locations. For example, while some tendon and ligament are subjected to low-magnitude, low-frequency, short-duration, and uniaxial loads, other tissues may experience high-force, repetitive, long-term, and/or multiaxial loading. Such variability of in vivo mechanical demands leads to tendon and ligament throughout the body that exhibit unique compositional and structural properties. In addition, obvious differences in composition/structure exist between mid-substance tendon and ligament tissue compared to enthesis tissue, where soft tendon or ligament transitions to unmineralized fibrocartilage and then mineralized fibrocartilage before transitioning into bone [10] (Fig. 54.2A). Each of the zones of the tendon and ligament–bone insertions contains specific compositional/structural features that enable force transmission across this complicated interface between dissimilar materials (Fig. 54.2B). While such adaptations to physiological loading is vital to proper tissue function, such variation also further complicates the already challenging endeavor of treating tendon and ligament injury and degeneration since there are unlikely to be any universal or one-size-fits-all solutions for repairing or reconstructing damaged connective tissues [10,21–23]. Since proper mechanical function is the key to success, tissue engineering strategies must seek to recapitulate the compositional, structural, and mechanical properties of tendon and ligament, including properties unique to specific anatomical locations and distinct loading conditions.

Requirements for a tissue-engineered tendon/ligament

A tissue-engineered tendon or ligament must be compatible with the physiologic environment where it will reside, macroscopically meet the anatomic demands of the tissue it replaces, and provide a hospitable microenvironment for a cell population to regenerate [24] (Fig. 54.3A). For these reasons, the selection of scaffold material for a tissue-engineered construct typically consists of (1) natural materials that exist in the native tendon and ligament ECM, (2) synthetic materials that are biodegradable and can eventually be replaced by ECM deposited by a native or new cell population, or (3) some combination of natural and synthetic materials that can impart advantages of

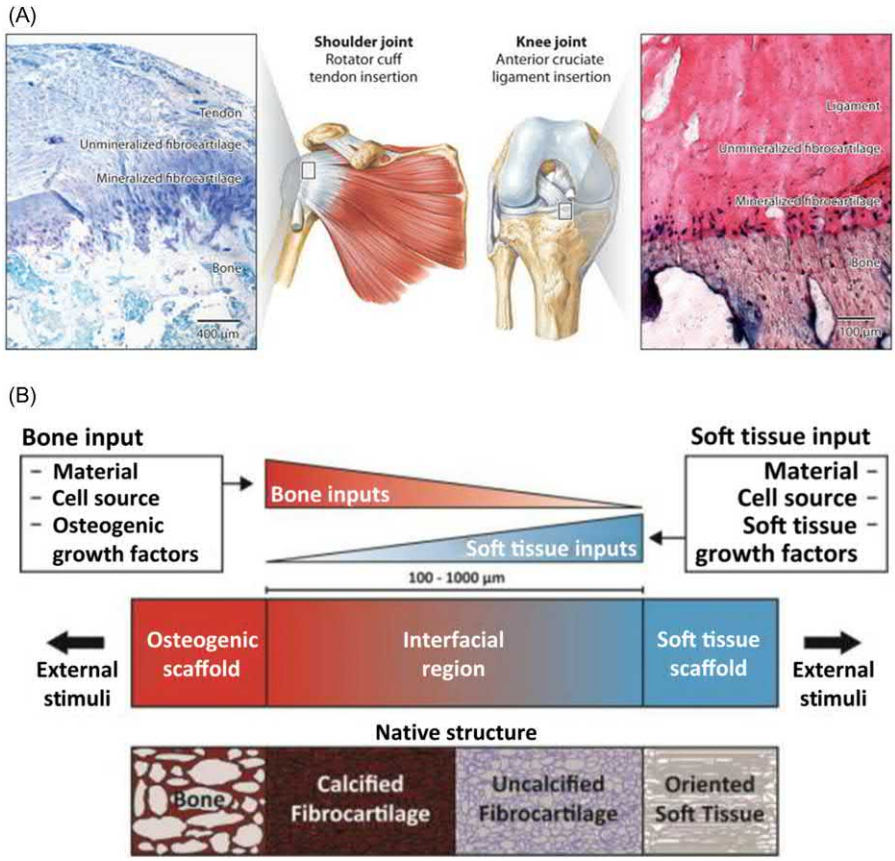

FIGURE 54.2 Considerations for engineering tissue entheses. (A) Histological images of soft tissue-to-bone insertions. Tendon (left) and ligament (right) entheses are characterized by transition from tendon or ligament, respectively, to unmineralized fibrocartilage and mineralized fibrocartilage before inserting into underlying bone. (B) Schematic depicting engineering considerations when creating a tissue-engineered soft tissue-to-bone insertion. *Reprinted with permission from (A) Lu HH, Thomopoulos S. Functional attachment of soft tissues to bone: development, healing, and tissue engineering. Annu Rev Biomed Eng 2013;15:201–26 [20] and (B) Boys AJ, McCorry MC, Rodeo S, Bonassar LJ, Estroff LA. Next generation tissue engineering of orthopedic soft tissue-to-bone interfaces. MRS Commun 2017;7(3):289–308.*

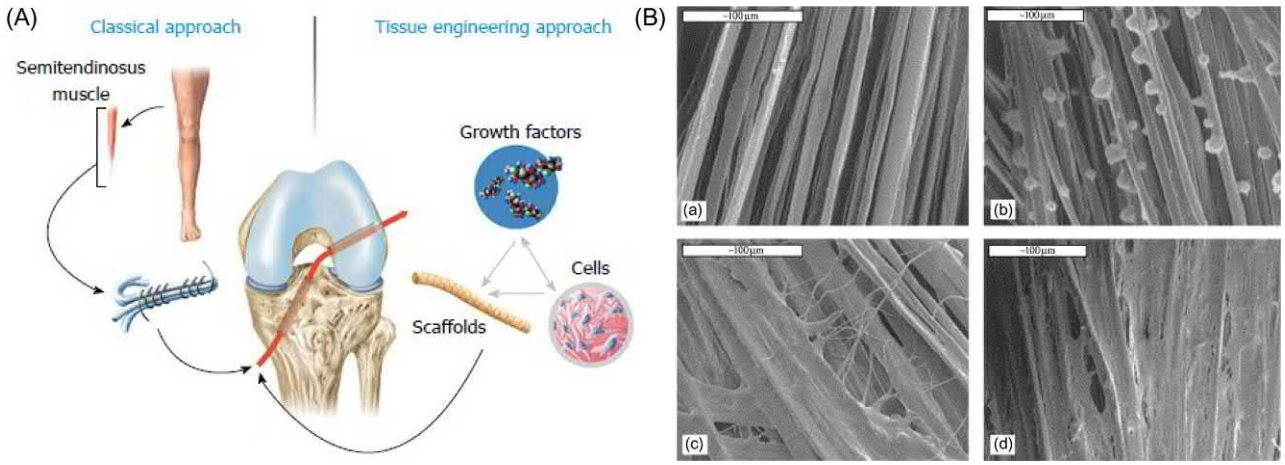

FIGURE 54.3 Representative paradigm for tendon and ligament tissue engineering. (A) A torn ACL is classically repaired using an autograft. Tissue-engineered constructs can be fabricated through combination of a scaffold, cellular component, and growth factors to achieve tissue regeneration. (B) Bone marrow stem cells (BMSCs) seeded onto a silk fibroin scaffold for ACL tissue engineering: (a) acellular scaffold has a highly aligned microstructure to mimic the native ACL collagen fiber alignment; (b) BMSCs are seeded onto the scaffold; (c) cell population begins to proliferate forming a cell sheet; and (d) which covers the scaffold with deposited extracellular matrix by day 14. *ACL*, Anterior cruciate ligament. *Reprinted with permission from (A) Nau T, Teuschl A. Regeneration of the anterior cruciate ligament: current strategies in tissue engineering. World J Orthop 2015;6(1):127–36 [25] and (B) Altman GH, Horan RL, Lu HH, Moreau J, Martin I, Richmond JC, et al. Silk matrix for tissue engineered anterior cruciate ligaments. Biomaterials 2002;23(20):4131–41 [26].*

both types of scaffold [27–29]. As the fundamental structural feature of tendon and ligament are a highly aligned type I collagen fiber matrix, the chosen scaffold must be able to recapitulate the high tensile strength that the native microstructure imparts. In addition to providing mechanical strength directly, the scaffold will also influence the cellular behavior as the cell population within a tissue-engineered construct will respond to the structure of the scaffold (alignment, organization, fiber size, pore size, etc.). Thus scaffold properties should be inspired by the native organization of the tendon and ligament in order to elicit a proper cellular response [30,31]. The cell population itself must be biocompatible, capable of ECM interaction, and able to exhibit propensity to remodel the scaffold in a manner similar to native tissue. Often, the natural choice is to utilize the native tendon and ligament cell type (ligamentous fibroblast and tenocyte), but a plastic stem cell source (embryonic, adult, or induced pluripotent) can also be utilized and stimulated appropriately to differentiate into a desired cell phenotype. In addition, paracrine signaling between these cell types can be exploited in coculture approaches, as stem cells have been shown to secrete cryoprotective factors that can positively influence regenerative capacity of native cell types [32,33]. Tendon and ligament construct design must provide support for cellular remodeling after seeding to ensure tissue maturation, mechanical development, and eventual adaptation to the in vivo environment. Maturation is essential for the proper mechanical functioning of a tendon and ligament-engineered construct, as its primary role is to attenuate load. Stimulus resulting in matrix remodeling can be provided preimplantation via mechanical stimulation during culture using a bioreactor that can match the desired loading environment [34]. Construct maturation can also occur through application of appropriate growth factors (GFs) and other biochemical signals provided in vitro [28]. Construct design should also allow for postimplantation maturation, as the in vivo environment equilibrates with the scaffold through processes such as tissue ingrowth. Finally, a proper soft tissue-to-bone interface (i.e., enthesis) should also be established in a complete tendon and ligament tissue–engineered construct to promote proper integration with the host site [10,22,35,36]. Although difficult to engineer due to gradations in composition, structure, mineralization, and cell type, entheses provide a direct means of fixation and mechanical support as a tendon or ligament inserts into bone.

Scaffold

The choice of scaffold for tissue engineering applications is crucial to the success of a potential implant. As most cells in human tissues are anchorage-dependent and responsive to their local environments, scaffold composition, architecture, and bioactivity will directly influence cell adherence, behavior, and matrix deposition [31]. The scaffold also provides the initial mechanical properties of a construct; if a scaffold cannot survive the loading environment of the implantation site, proper maturation (mechanical or chemical) of the graft cannot take place [10,28]. The selected scaffold material can also, through modification or functionalization, act as a vehicle for

delivery of biochemical or GFs. As the scaffold effectively provides the backbone for the engineered construct, care should be taken to select and tune the properties of the scaffold to provide the best possible match to those of the tendon or ligament that will be replaced.

Composition/material selection: The primary constituent of tendon and ligament ECM is type I collagen, and as such, is often the protein of choice for scaffold material [27]. Collagen is simple to isolate through techniques such as acid extraction or pepsin digestion, and is intrinsically biocompatible [37]. Collagen-based scaffolds can be created as sponges through freeze-drying [38], as a hydrogel [39], electrospun into nanofibers [40], as well as numerous other forms. When fabricating collagen-based materials, properties such as shape, pore size, fiber alignment, density, and mechanical robustness can be tuned through a variety of fabrication procedures [37]. Cells seeded onto a collagen scaffold will actively adhere due to presence of integrin-binding domains [41], which can then actively remodel the matrix based on prescribed stimuli [42]. However, collagen scaffolds—particularly as a hydrogel—often fail to achieve sufficiently strong mechanical properties necessary to survive the load-bearing environment of a tendon or ligament after implantation [28].

Many other naturally occurring (noncollagenous) materials have been used as scaffolds for tendon and ligament tissue engineering. Silk fibroin was one of the first materials used for ligament tissue engineering [26,43] and shares many of the same benefits as type I collagen (Fig. 54.3B). Silk fibroin has mechanical properties comparable to that of native tendon and ligament (e.g., ultimate tensile strength, linear stiffness, and yield point [26]) when fabricated into structures such as cords, nets, and sponges [26,44]. Importantly, silk fibroin can also be formed in a manner that recapitulates some aspects of the hierarchical structure seen in the native collagen ECM in tendon and ligament [26], which may be important for structural support and mechanobiological signaling across length scales.

Tissue-engineered scaffolds can also be created for tendon and ligament applications from a number of natural hydrophilic polymeric biomaterials such as chitosan [45], gelatin [46], fibrin [47], alginate [48], and hyaluronan [49,50]. These types of biomaterials are often formed into hydrogel scaffolds, consisting of a small portion of polymer with a very high percentage of water. A highly hydrated structure can be advantageous for tendon and ligament tissue engineering by providing a suitable microenvironment for cell suspension as well as flexibility in construct design [50]. However, hydrogels on their own typically lack mechanical strength compared to other scaffolding options, but can be reinforced with fibrous materials via a composite scaffolding approach [51].

Another popular method of scaffold fabrication is decellularization of native tendon or ligament tissue [52]. Elimination of the native cell population produces a scaffold with preserved biomechanical properties, matrix organization, and mature cross-links, yielding a scaffold that is well suited to perform in vivo [53]. Decellularization can also preserve native tendon or ligament entheses [10,53], which helps make implantation more effective, improve mechanical performance, and eliminate the technically challenging requirement of engineering a new soft tissue-to-bone transition [21,36,54]. Cells are able to repopulate decellularized grafts to provide bioactivity, but can be limited by the degree to which they can infiltrate into the decellularized scaffold [55]. In addition, lack of complete decellularization of a tissue can result in an inflammatory response upon implantation [56], which may limit overall success.

The necessity of selecting a scaffold material that is both biodegradable and mechanically robust has led tissue engineers to fabricate tendon and ligament constructs out of synthetic materials. The motivation for using synthetic materials is to provide a more stable initial structure than natural materials can allow, permit cellular infiltration, then subsequently degrade at a rate similar to that at which the cell population can produce and deposit new ECM [28,29]. As these materials are fabricated, their biophysical, biochemical and structural properties are inherently tunable and can be manipulated to direct cellular function and behavior [57]. Some common materials used for tendon and ligament tissue engineering include polyglycolic acid, polylactic acid (PLA), polycaprolactone (PCL), and polyethylene glycol (PEG) diacrylate [28,58–61]. One primary concern with using these synthetic materials is the possibility of a negative reaction of the cell population. Modification of the scaffold (e.g., addition of integrin-binding motifs) is often needed to ensure cell attachment and proliferation; such effects may be due to high hydrophilicity of some polymers [62]; however, this is not consistent for all synthetics [59]. As selected polymers are typically biodegradable, care must also be taken to ensure that the degradation rate is not too rapid to outpace corresponding adequate tissue ingrowth after implantation or that the degradation process does not produce acidic byproducts [59].

Structure/architecture: Fabrication of scaffolds can be performed with a variety of techniques that allow for morphological aspects to be tuned as well [63,64]. Processes such as electrospinning allow for directed fiber alignment of a scaffold, which has been shown to influence cellular responses such as motility and ECM deposition [65]. Gradients in scaffold fiber alignment have also been shown to aid in recapitulation of characteristics of soft tissue-to-bone interfaces [10,66,67]. Other microstructural features (e.g., pore size) can be controlled using

techniques such as salt leaching [66,68], freeze-drying [69–71], electrospinning [72], and weaving or braiding [59,73]. In addition, scaffold materials can be functionalized or conjugated with GFs to further direct construct maturation [57]. Finally, although synthetic or natural materials have been historically used separately for replacing damaged tendon and ligament, recent work has created composite scaffolds that are able to encapsulate the advantages of each type of material [28]. Composites can also be used in engineering scaffolds for the soft tissue-to-bone interface, as they can help provide appropriate regionally dependent compositional and structural stimulus to a cell population when recapitulating a multitissue environment [74]. Furthermore, proper selection of scaffold material and design of architectural components are critical in both providing biomechanical support and development of a tendon and ligament construct microenvironment capable of regeneration.

Cell

Cell-based strategies have been widely explored in tendon/ligament tissue engineering and have great potential for developing novel translatable therapeutic approaches. Two categories of cells have been the main candidates for tendon/ligament tissue engineering, namely differentiated cells (such as tenocytes and fibroblasts) and mesenchymal stem cells (MSCs). MSCs are capable of differentiating into distinct cell types, secreting trophic factors, and eliciting minor immune responses, thus making them a potentially better source for clinical application compared with differentiated cells. MSCs are commonly delivered to the injury site by isolated cell injections; however, this method is associated with significant cell loss and low cell-adhesion efficiency. Cell sheet technology, an alternative approach of delivering cells, that overcomes disadvantages of cell injections and allows readily cell-host tissue integration, has been recently developed (Fig. 54.4) [75]. Human rotator cuff–derived cell sheets have been shown to improve enthesis healing after infraspinatus tendon repair in a rat model [76]. Similarly, the utilization of human ACL-derived cell sheet in a rat ACL reconstruction model promoted tendon-to-bone healing in terms of graft maturation, proprioceptive recovery, and mechanical strength [77]. In addition, recent research using a large preclinical canine model demonstrated superior enthesis healing after rotator cuff repair augmented with a multiphasic scaffold and bone marrow–derived mesenchymal stem cell (BMSC) sheet [78]. Given these encouraging results, cell sheet technology may serve as a new strategy for improving tendon/ligament repair and regeneration on patients.

Increasing evidence has revealed that ECM microenvironment cues play a pivotal role in determining stem cell fate [79–82]. Decellularized scaffolds or bioimprinted materials that possess or replicate the natural tendon ECM microenvironment can support MSCs distribution, direct MSCs alignment, and induce MSCs tenogenesis [80,82]. Furthermore, the combination of mechanical stimulation and inherent ECM cues is able to provide sufficient signals for guiding stem cell differentiation toward a tenogenic/fibroblastic pathway without exogenous chemical stimuli [81,83]. Therefore seeding cells on materials that recapitulate native tendon/ligament ECM cues together with optimized mechanical stimuli would yield biomimetic functional substitutes for promoting tendon/ligament tissue regeneration and reconstruction.

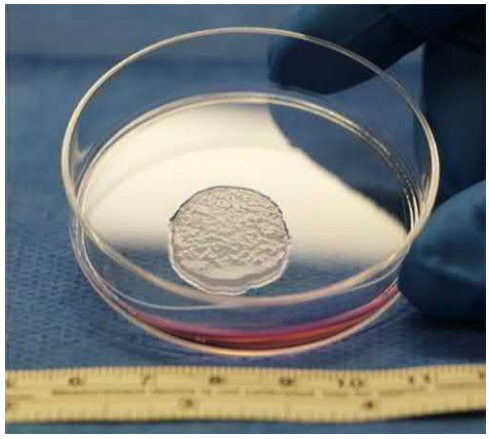

FIGURE 54.4 An intact BMSC sheet was detached from the dish and ready for implantation.

Various sources of MSCs, including bone marrow, adipose tissue, muscle, tendon, and bursa, have been investigated for tendon/ligament tissue engineering with overall promising results in preclinical studies [84,85]. Among different tissue-derived MSCs, BMSCs are currently the preferred cell source in clinical settings. In a clinical study with up to a 10-year follow-up, patients that received autologous BMSCs injection during single-row arthroscopic rotator cuff repair had better healing rate by 6 months and higher tendon integrity rates at the final follow-up [86]. In addition, recent studies have shown the beneficial effects of bone marrow stimulation on reducing retear rates among patients undergoing surgical repair of large to massive rotator cuff tears [87,88]. Through bone marrow stimulation that is performed by creating multiple holes at the footprint, the endogenous BMSCs are recruited and released from the proximal humerus and subsequently infiltrate the repaired tendon and contribute to postoperative enthesis healing. These results emphasize that autologous BMSCs, either by implantation or stimulation, play a positive role in rotator cuff surgical treatment. Conversely, a clinical study using stem cells in ACL reconstruction suggested that intraoperative autologous BMSC application to the femoral tunnel did not

accelerate graft osteointegration at 3 months based on magnetic resonance imaging (MRI) assessment [89]. In the future, more clinical studies evaluating other tissue-derived MSCs are needed to identify the best cell source for tendon/ligament regeneration in humans.

Bioactive factors

The past decade has witnessed an upsurge of interest in using GFs and platelet-rich plasma (PRP) to improve tendon/ligament healing or as adjuvants for surgical intervention. A variety of GFs, including transforming GF, bone morphogenetic proteins (BMPs), fibroblast GF, platelet-derived GF, granulocyte-colony stimulating factor, vascular endothelial GF, matrix metalloproteinase inhibitors, have been reported to exert positive effects on tendon/ligament healing in numerous laboratory and animal studies [84,85,90]. In contrast to individual GFs, PRP contains various types of GFs and has been extensively studied in the field of tendon/ligament tissue engineering. PRP can be administered intraoperatively or postoperatively, directly or indirectly to the injury site. Using small animal models, it has been shown that autologous PRP administration resulted in improved tendon-to-bone healing histologically and mechanically in surgical repair of either acute or chronic rotator cuff tears [91,92]. Similarly, PRP in combination with BMSCs or bone substitute enhanced graft-bone healing after ACL reconstruction as determined by imaging, histology, and biomechanical testing [93,94]. With respect to large animals, researchers found that multiple intra-articular injections of PRP could promote ACL healing, improve limb function, and reduce pain in a canine ACL deficiency model [95]. In addition, repair of large and retracted cuff tendons using PRP hydrated bone matrix led to increased mechanical strength, improved histology and MRI scores in a canine shoulder model [96].

Interestingly, while the laboratory and animal studies have shown overall favorable effects of PRP application, the clinical results on PRP remain controversial regardless of rotator cuff repair or ACL reconstruction. In a comparative cohort study assessing arthroscopic repair of large or massive cuff tears with or without leukocyte−platelet-rich plasma injection, no appreciable difference was found between groups in terms of tendon healing and functional outcomes at a minimum 2-year follow-up [97]. In a randomized clinical trial (RCT), the use of PRP perioperatively in patients undergoing ACL reconstruction failed to prevent tunnel widening based on CT scanning although similar clinical outcomes were observed at 3 months post-surgery [98]. Furthermore, several RCTs have suggested that there was no significant improvement in structural integrity and functional outcomes in patients receiving PRP as adjuvants for rotator cuff repair [99−102]. A recent systematic review also revealed that PRP might show promise in accelerating graft maturation after ACL reconstruction, but its beneficial effects on clinical outcomes has not been proven yet [103]. The discrepancies between experimental and clinical studies could be attributed to the native differences between animals and humans; the timing, concentration, and formulation of PRP; and the relative short follow-up time in clinical trials. The true clinical efficacy of PRP in tendon/ligament healing could be answered by future high quality RCTs with a long-term follow-up.

Over the past few years, the secretome of MSCs has gained increasing attention and has emerged as a potential cell-free strategy in regenerative medicine. It has been revealed that MSCs-derived secretome promoted human tendon cell viability in vitro and improved enthesis healing when combined with an electrospun keratin scaffold in surgical repair of massive rotator cuff tears in rats [104]. Among the numerous contents of MSCs-derived secretome, exosomes are believed to be the primary agent mediating the therapeutic effects on tissue repair or regeneration. Exosomes are nanosized vesicles containing RNAs and proteins which critically involved in cell−cell communication (Fig. 54.5). A recent study has shown the

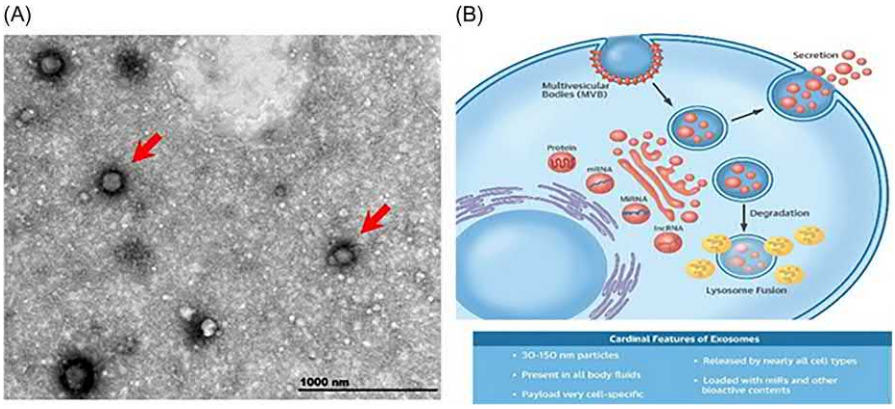

FIGURE 54.5 (A) The morphology of human plasma-derived exosomes under TEM. (B) The basic features of exosomes. *TEM*, Transmission electron microscopy. *Reprinted with permission from Journal of the American College of Cardiology 71(2), 2018.*

efficacy of human MSCs–derived exosomes in enhancing periodontal ligament cell proliferation and migration, as well as improving periodontal tissue regeneration in a rat model [105]. Despite the fact that benefits of MSCs-derived exosomes have been evaluated in vascular, muscle, and bone regeneration [106–108], its role in tendon/ligament tissue engineering has not yet been explored. With the growing interest in exosome-based therapy, future studies are warranted to elaborate its function on tendon/ligament healing and to develop novel cell-free treatment for clinical translation.

Three-dimensional bioprinting and bioink

In general, an engineered tissue or organ can be constructed with two strategies: bottom-up and top-down [109–112]. For bottom-up strategy, a tissue is built from materials at molecular or nanolevel to micro or macroscopic level. For top-down strategy, cells and bioactive molecules are seeded into a macroobject to make a tissue construct. Three-dimensional (3D) printing is a technology that can construct a tissue either from microscale in cellular level (bottom-up) or volume porous scaffold (top-down) [113].

3D printing is a kind of additive manufacturing. The driving idea was borrowed from "machine" operation, where a lathe can be numerically controlled to move in *X*, *Y*, and *Z* direction by a computer. The computer can "talk" to the lathe using "G-code" for communication. For a 3D volume or block process (manufacturing) in printing, an STL, an abbreviation of "stereolithography," with 3D point-line-surface information is a commonly adopted format and created by computer-aided design software. Thus a slicing program is needed to "cut" the 3D block into paper-like pieces where their dimensional (location) information is stored in G-code in each plane. Thus a 2D "paper" can then be stacked into a 3D volume (block). The sliced "paper" can be tuned in its thickness from a few microns to hundreds, which depends on the printing's resolution. The materials used for bioprinting are named "bioink [113]."

In general, there are four types of bioprinting methods, including extrusion-based, inkjet, stereolithography (SLA)-based, and laser-assisted. For extrusion, one of the most popular bioprinting technologies, bioink is loaded in a container, commonly, a syringe tube. Air pressure or screw-driven plate pushes the bioink to go through an orifice, which can be tuned in size for the considerations of bioink viscosity, deposition rate, and the printed objects size and accuracy (Fig. 54.6). The temperature effects could also be added in the printer for thermal sensitive polymers and melting plastics. Once bioink for extrusion printer gets through a small orifice, the ink experiences shear-thinning phenomena (Fig. 54.7). Under a high

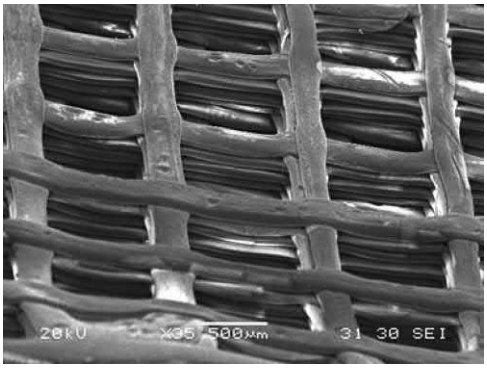

FIGURE 54.6 A printed hydrogel scaffold with controlled pattern size, pitch width, line width, and the number of layer.

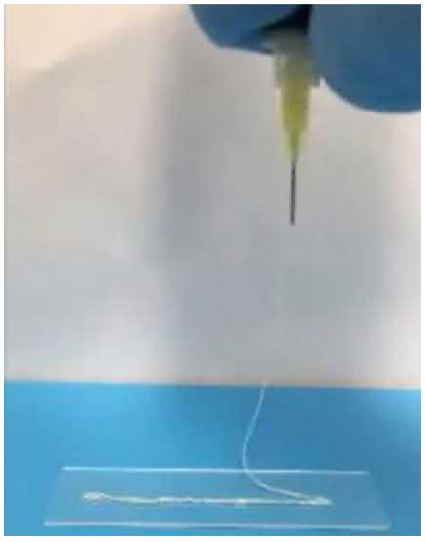

FIGURE 54.7 Bioink's shear thinning and viscosity are critical for a successful extrusion and deposition to form 3D pattern. *3D*, Three-dimensional.

pressure and shear stress, its viscosity will decrease in the orifice but will go back to the original after the ink is printed out. The procedures perfectly simulate the bioink passing through small orifice with high pressure and shear ratio. Viscosity is also critical for bioink's deposition onto the printing substrate. Inks with a low viscosity are not favorable for layer-by-layer constructs, while high viscosity inks perform well since under a stroke of extrusion the ink should be cleared away from printing head for next G-code input. Thus a rheological test can be conducted to characterize the viscosity mechanics of various bioinks under different shear rates and temperatures.

The inkjet approach to bioprinting (Fig. 54.8) is like an "office-use" printer with bioink in their cartridge classified with continuous-inkjet bioprinting, electrohydrodynamic jet bioprinting, and drop-on-demand inkjet bioprinting from driving mechanisms of thermal, piezoelectric, and electrostatic. From the perspective of

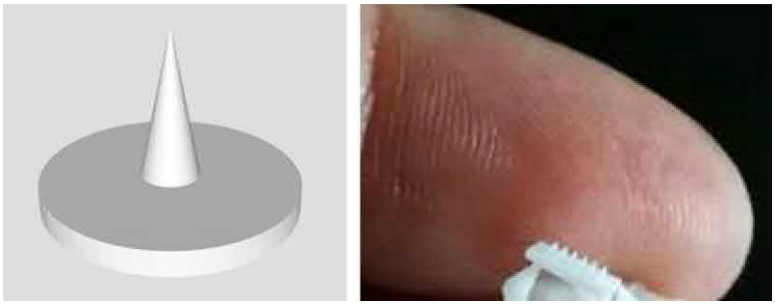

FIGURE 54.8 SLA printed high-resolution microneedles.

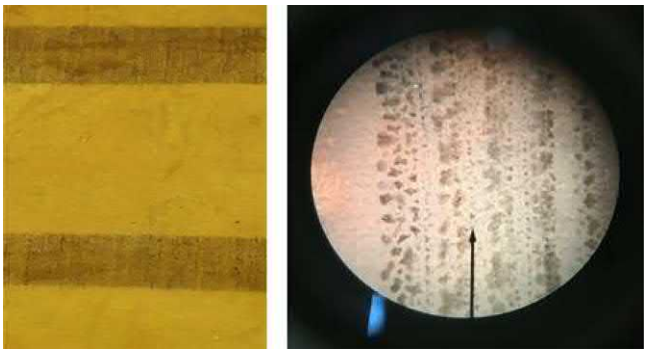

FIGURE 54.9 Inkjet printed graphene oxide–gelatin pattern with high-resolution finer line inside (right:under microscopy).

efficiency and convenience, inkjet printing has limited popularity compared with extrusion-based printers. In addition, the viscosity of available bioinks is not as diverse as in extrusion printers. Inkjet-based printing is a noncontact printing technique in which droplets of dilute solutions are dispensed, driven by thermal, piezoelectric, or microvalve processes.

SLA is a technology to use light with a specific wavelength to polymerize molecular chains and/or to make crosslinks to form polymer networks (Fig. 54.9). After polymerization and cross-linking the formed bulk system will not be dissolvable in water. Noncross-linked portions will be sacrificial layers that can be washed away in water or other solvents, while the remaining material will be printed in desired patterns. In general, bioink precursors have double bonds (e.g., vinyl), which can be activated for free-radical polymerization by light (e.g., wavelength of 365 nm). One majorly used and verified safe initiator is Irgacure2959 (Ciba), while some commonly used bioinks are acrylate-carried biomaterials (e.g., PEG, gelatin, chitosan, F127, acrylic acid, and acrylic amide) and methacrylate (MA) derivatives, etc.

Developed decades ago, laser-induced forward transfer (LIFT) can be used in 3D printing with high-resolution deposition of bioink. Pulsed-laser evaporation for direct writing has been used for cell printing. The mechanism is based on high powerful pulse laser and two glass slides, with one as energy absorption and the other containing cells. LIFT has high resolution but is also with high cost.

Bioink inspired from ligament and tendon structures

A tendon is composed of toughly packing self-assembled and paralleled multiscaled collagen fibers. This connective tissue bridges muscle to bone to sustain cyclic mechanical loadings of tension, compression, torsion, and shear. Similar to tendons in mechanics, ligament connects bone to bone. 3D bioprinting tissue could be used as a model to study musculoskeletal related disease and to screen drug molecules. Gelatin-MA was printed on a microplate for musculoskeletal tendon–like tissues on postholder cell culture inserts in 24-well plates. Human primary skeletal muscle cells and rat tenocytes were cocultured around the posts. Different printing patterns were used to demonstrate related gene and protein expressions, which could be used as a screening platform [114]. ACL is commonly reconstructed with tendon grafts following injury. Tissue-engineered implants can be printed in a thin-walled cylindrical mesh (Fig. 54.10), which can be used to enhance the strength for ACL reconstruction as an internal brace. The printing ink was composed of PCL, poly(lactic-*co*-glycolic acid) (PLGA), and β-TCP (tricalcium phosphate) under a pneumatic pressure of 500 kPa for deposition [115]. However, the integration between implant and bone had potential complication due to insufficient bone filling. Thus in a subsequent study, 3D printed cylindrical mesh was coated with recombinant human BMP 2 (rhBMP-2) with poly(propylene fumarate) as bioink; results demonstrated significantly increased pullout strength [116].

Until now, for 3D printed functional ligament and tendon, the research mostly focused on using thermal plastic polymers (such as PCL, PLA, and PLGA), natural hydrogels (such as gelatin, chitosan, alginate, and fibrin) and some ceramic materials (such as hydroxyapatite and β-TCP). In general, plastics and ceramics contribute to stiff phase and hydrogels contribute to soft phase. Meanwhile, cells and therapeutic agents can be added inside. However, a tough but flexible structure is able to mick ligament and tendon is still a challenge. For 3D printing ligament and tendon, the key is about how to deposit bioink with toughness, mimetic structures, and

FIGURE 54.10 3D printed cylindrical tough mesh coated with hydrogel. *3D*, Three-dimensional.

cell-compatible environments. Toughness means how much energy the material can absorb before failure. In order to enhance mechanical strength and fatigue resistance, multiple methods have been considered. Physical bonds can be introduced [e.g., polyacrylic acid, polyacrylamide (PAM), cellulose, and mussel-inspired dopamine]. On the other hand, a chemical double-network hydrogel can be designed with an elastic phase on chemical cross-linking and a dissipative phase on physical cross-linking. For instance, PAM gel with appropriate concentrations of monomer acrylamide and methylenebisacrylamide cross-linker is a superelastic gel, and alginate cross-linked with a cationic chelator Ca^{2+} is a physical gel. Upon mechanical impact, the PAM phase experiences fast deformation, and the stored elastic energy can be consumed by sacrificing physical cross-linking, which is reversible [117]. Liu et al. demonstrated gelatin-MA, a chemical and biocompatible gel reacted with tannic acid, can lead to a strong, tough, compressible, and stretchable gel with self-healing even underwater [118–122]. Electrospun short nanofibers of PCL, PLA, polyurethane, and natural polymers such as chitosan, alginate, collagen (type I), and gelatin can be mixed with ink–gel for printing. Nanofibers are pushed through printer's orifice (normally a long needle with regular round shape). The pressure and shear stress will promote these short nanofibers arranged to follow the flow direction. As a result, the nanofibers can have an orientation with alignment tendency. Upon light cross-linking, a tough scaffold with aligned fibers can be produced, for example, electrohydrodynamic jet printed out multilayered microfibrous films with longitudinal orientation fibers [123]. The film can be rolled up into a sleeve-like structure with high mechanical strength, which also enables migration of human tenocytes into the film with enhanced cellular activity.

In addition, some inorganic micro- to nanostructures can be used for mechanical enhancement of 3D printing. Cellulose nanofibers (or nanocrystal) are natural polymers with high aspect ratio and high stiffness, which can be included for tough and aligned scaffolds. Besides, hydroxyl and/or carboxylate functional groups provide multiple opportunities for modification (e.g., cellulose can be oxidized to produce aldehyde groups). It offers a convenient approach to working with the amine groups of gelatin for a bioink due to the Schiff base formation. Similar to cellulose nanocrystal, carbon nanotube and graphene oxide (Fig. 54.11) can also be used in bioinks as mechanical enhancers.

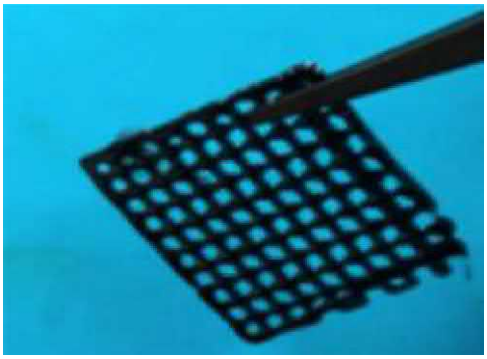

FIGURE 54.11 3D printed PAM with chemical crosslinking and graphene oxide with physical crosslinking composite hydrogel. *3D*, Three-dimensional, *PAM*, polyacrylamide.

Tissue engineering tendon and ligament in clinical application

A typical tissue-engineered tendon and ligament should include scaffold, cells, and bioactive factors. However, this ideal engineered tendon combined with all three components has not been tested in clinical trials due to the barriers of the biological complexity for clinical applications. As the tendon and ligament is acting as a biological cable to move and stabilize the joints, engineered tendon and ligament scaffolds alone have been widely used in the clinic to treat tendon and ligament injuries. There are mainly three clinically used types of scaffolds, including native tendon tissue, other connective tissues [small intestine submucosa (SIS), fascia lata, etc.], and synthetic material tendon/ligaments. Native tendon scaffolds are the most common form for clinical applications, which include autograft, allograft, and xenograft. The tendon autograft to replace either damaged tendon or ligament is the gold standard to treat tendon and ligament defect in clinical practice. However, due to limited resources and

donor morbidity of the autograft tendons, allograft and xenograft tendons, or other tissues, have been utilized increasingly in recent decades. In order to reduce immunogenicity of the allografts, a lot of biologic scaffolds have been derived using the decellularization technique. Currently, the clinically approved connective tissue scaffolds include allograft (acellular human dermis) and xenograft (bovine pericardium, porcine SIS, porcine dermis) [124–127]. In general, the allograft or hybrid graft could provide satisfactory clinical outcomes [125,126,128–135]. When compared to the repairs without grafts, the use of grafts provided superior tendon healing [125–127]. However, the autograft still showed better results compared to allograft [136]. It is important to pay close attention to the processing procedure of the allograft because this could affect clinical outcomes. Many studies found that the use of nonirradiated or nonchemically treated allografts could achieve comparable outcomes to those repairs with autografts [128,129]. As for the xenograft, many studies have reported the high risk of inflammatory reaction, which refrain their utilization in clinic [125,127]. Iannotti et al. demonstrated the use of SIS resulted in an inferior healing rate compared with repair without augmentation [137]. Inflammatory reactions were observed in the patients using SIS augmentation [137]. Phipatanakul and Petersen reported complications of SIS, which included one infection and two localized skin reactions [138]. Walton et al. also confirmed a severe inflammatory response to SIS [139]. Lots of synthetic grafts have been proposed since the beginning of the 20th century. The materials for synthetic grafts include polypropylene terephthalate, poly-L-lactide acid, and polycarbonate. The synthetic patches show initial promising biomechanical properties [126]. However, the high failure rates have been confirmed among most of the artificial grafts used for ACL reconstruction. The failure rate after an ACL reconstruction, using Darcon, ranged from 20% at 2 years postoperatively up to 44% at 9 years [140,141]. The main concern about using synthetic grafts is the risk of infection and adverse host reaction [142]. More randomized controlled trials are needed to confirm their long-term clinical outcomes.

Bioactive factors, including GFs and small molecules, play an important role in tendon/ligament tissue engineering. With many preclinical models, the bioactive factors have been well proved to enhance tendon/ligament healing [143–146]. However, these results could not be directly translated to clinic practice. The main challenges have to be overcome such as the selection of optimal GF(s) or combination, identification of the most efficient stage and duration of delivery, and the design considerations for the delivery device [143]. Many researchers endeavor to figure out the dose concentration and feasibility of bioactive factors when applied to repair tendon/ligament in clinic [147,148]. Ide et al. showed local rhBMP-12 on an absorbable collagen sponge could be an adjuvant therapy for rotator cuff repair [147]. Oh et al. investigated the effect of recombinant human growth hormones on the outcomes after arthroscopic rotator cuff repair [148]. There was no statistically significant improvement in healing or outcomes related to the treatment of human hormone after repair. Therefore further, larger, and multicenter clinical studies should be conducted. Platelet-rip plasma (PRP) products have been well investigated in the recent decades. Wang et al. found that the PRP injection after rotator cuff repair did not enhance the early tendon healing and functional recovery [101]. Many other studies also confirmed that the use of PRP would not improve the rotator cuff healing [149–151]. However, for treatment of patellar tendinopathy using PRP, the clinical outcomes are controversial. Based on a systemic review of 15 clinical trials, Jeong et al. showed promising potential of PRP to restore patients to their daily work, living, and sports [152]. Nonetheless, it is hard to draw a clear conclusion for the effectiveness of PRP treatment on patellar tendinopathy [152]. With regards to ACL reconstruction, there was no proof that the use of PRP could enhance the ACL surgery clinical outcomes [103].

The MSC-based tissue engineering approach has emerged as a new therapy for tendon/ligament repair. The autologous stem cells utilized in the clinic are mainly bone marrow stem cells (BMSCs) or adipose-derived mesenchymal stem cells (ADSCs). Both BMSCs and ADSCs showed promising clinical results in rotator cuff injury [86,153,154]. Hernigou et al. demonstrated that the healing outcome of rotator cuff repair was significantly augmented with BMSCs [86]. Kim et al. revealed that the injection of ADSCs loaded in fibrin glue could significantly decrease the retear rate [154]. There were, however, no clinical outcome differences after a 28-month follow-up [154]. Although lots of preclinical studies showed benefits of MSC in the repair of ACL in animal models, only one clinical study, conducted by Silva et al., reported that adult noncultivated BMSCs did not seem to accelerate graft-to-bone healing [89].

In conclusion, tissue-engineered tendon and ligament have a high potential for clinical application due to a large amount of clinical demand. However, there is limited consensus in terms of scaffolds, bioactive factors, and stem cells in clinical trials. Further, basic science research for revealing mechanism and large animal models for translational research are critical for the successful clinical trials.

Summary

Tendons and ligaments are dense soft connective tissues that connect either muscle to bone (tendon) or bone to bone (ligament) to mobilize or stabilize the joints. Almost

all skeletal muscles need tendons to attach to bones, and all joints rely on ligaments for their stabilization and function. Loss of tendon or ligament integrity impairs muscle, bone, and joint function. Therefore tendon ligament functional restoration after injury is crucial. Due to anatomic and functional characteristics, tendon and ligament are the most vulnerable tissues suffering from injury in the musculoskeletal system, including acute and chronic injures. Furthermore, tendon and ligament have less vascularity and cellularity compared to other musculoskeletal connective tissues, such as bone, that lead to a slow and incomplete tissue healing with fibrotic scar formation. Treatment of tendon and ligament injuries remains a significant clinical challenge. Complications during the healing process are also high, such as retear of the rotator cuff repair, which has been reported as high as up to 90% [155–157]. Tissue engineering in the tendon and ligament field has been increasingly investigated recently in both experimental and clinical studies. As the tendon or ligament is a connective tissue, the mechanical strength, especially tensile properties, is the most important parameter when the engineered tendon or ligament is constructed. Thus the scaffold of the engineered tendon or ligament becomes crucial. Although many scaffolds, including native tissues or synthetic materials, have been used for tendons and ligament tissue engineering, the native tendon seems a good biomaterial for the tendon and ligament scaffold. However, as the tendon is a dense connective tissue, the revitalization or recellularization is challenging. Some technologies to increase the cell infiltration, such as multislicing or multislide, have been recently studied in animal models with promising results [158–160]. However, preclinical long-term studies would be necessary before clinical trials could be pursued. Cellular therapy and bioactive factors, especially PRP, have been used for tendon and ligament repair and regeneration clinically. However, there are no clear conclusions that can be determined from the limited number of clinical trials to date. More extensive, well-executed randomized control trials are needed in the future. Therefore in the next decade, tissue engineering in tendon and ligament area will remain a hot topic in both clinical trials and experimental studies. We hope there will be a breakthrough in the development of tissue-engineered tendon and ligament that could be translated successfully in clinical practice and commonly accepted by the clinical practitioners.

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Chapter 55

Skeletal tissue engineering

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Introduction

With an aging global population, the socioeconomic and biomedical burden from musculoskeletal disease remains high. Compared to the period from 1990 to 2007 the percentage change of years lived with disease over the past decade have continued to increase [1], and musculoskeletal health issues have substantially contributed to nonfatal health loss and lower quality of life. With an increasingly elderly population, the number of high risk osteoporotic fractures has also been estimated to double by 2040 [2]. In light of this, developments in materials science and bioengineering, stem cell biology, and molecular biology have begun to converge on strategies to accelerate bone formation through tissue regenerative approaches and this offers an exciting new option to address the reconstruction of osseous defects.

The clinician is confronted with an array of large nonhealing bone defects resulting from trauma, resection of malignancies, nonunion of fractures, and congenital malformations. Autografts remain the best option for reconstructing bony defects because they provide osteogenic cells, osteoinductive factors, and a lattice needed for bone regeneration. They can be obtained from a patient's calvarium, ribs, iliac crest, distal femur, greater trochanter, or proximal tibia. Autografts, however, subject the patient to inherent risks of surgery during harvest, with recent large studies following iliac crest bone graft harvesting, for example, reporting acute gait abnormalities in 45.7% and persistent donor site pain in over 3% [3]. Furthermore, autografts are a relatively scarce resource and are limited by the amount of bone that can be removed. In situations where autografts are not sufficient or possible, allogeneic bone from donors or cadavers may be used. Allografts, however, may be accompanied by the risk for disease transmission, immunologic rejection, and graft-versus-host disease. To minimize the risk of disease transmission and contamination, there are strict guidelines in place for allogenic bone providers; however, there are few guidelines outlining donor eligibility [4].

As an alternative, synthetic materials such as hydroxyapatite (HA), polyetheretherketone, polymethylmethacrylate, and titanium have been used as bone substitutes to repair skeletal defects. Each, however, has its own associated problems [5], and the choice of biomaterial frequently relies on surgeon preference. However, when choosing, it is important to weigh considerations, including biocompatibility, osteointegration, mechanical resistance, aesthetic results, cost, availability, and infection risk of each material type. Unfortunately, comparing outcomes of each material has been complicated by variables such as their use in different defect sizes, the quality of surrounding bone, preexisting infection, radiation history, and age of patient. The wide range of options available for osseous reconstruction is indicative of the many advances made in biomaterials, and recent developments in biotechnology utilizing computer 3D analysis has allowed for patient-specific synthesis of biomaterials and optimization of surgical planning resulting in reduced operating time and complication rates [6]. Importantly, the plethora of options also reflects the inadequacies of any single method. As such, tissue engineering holds great promise to adequately address the issue of repairing bone defects. The delivery of osteogenic cytokines is already employed clinically, specifically bone morphogenetic proteins (BMPs), for the induction of bone regeneration. The ultimate translational goal, however, is to deliver both osteogenic cells and cytokines via a biologically active scaffold, in order to heal a bony defect in an accelerated fashion.

This chapter will begin with an overview of distraction osteogenesis, a modality that translates directed mechanical force into endogenous bone formation, to emphasize

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the important role of the mechanical environment in bone formation and to highlight some of the growth factors that play a role in inducing bone formation. Emerging data in this field have also highlighted the significance of recapitulating developmental programs in bone formation leading to an evolution in regenerative paradigms. This chapter will now discuss recent advances in our understanding of skeletogenesis and the implications these discoveries have on cell-based strategies for bone tissue engineering. Finally, use of osteogenic cytokines and scaffolds to promote bone reconstruction will be examined, with an emphasis placed on recent discoveries.

Distraction osteogenesis

Distraction osteogenesis provides a model for the study of endogenous bone formation in large skeletal defects. First described by Alexander Codivilla in 1905 for limb lengthening and later codified by Gavril Ilizarov in the 1950s, distraction osteogenesis represents an endogenous form of bone tissue engineering [7,8]. New bone is formed during the process of separating osteogenic fronts with gradual but constant mechanical force. As a first step, an osteotomy is usually performed on the bone of interest, followed by application of rigid fixation. This is followed by a variable latency period of several days, during which a soft callus forms between the two osteogenic fronts. Gradual distraction is then applied, followed by stable fixation/consolidation, until a robust osseous regenerate is formed. It is thought that the mechanical forces applied by distraction can contribute to bone formation by induction of cytokines that guide mesenchymal cells in the bony gap to differentiate along an osteogenic lineage. Distraction osteogenesis not only triggers bone formation but also stimulates local angiogenesis as part of the process [9].

Research has been devoted toward characterizing the association between stress and strain patterns with bone formation. Correlating tensile force measurements with histology, Loboa et al. demonstrated that the greatest amount of bone formation occurs during active distraction, the period of greatest strain [10]. Loboa went on to further characterize the forces of distraction, using finite element analysis models created from three-dimensional computed tomography image data of rat mandibles at different phases of distraction osteogenesis [11]. The models described patterns of moderate hydrostatic stress within the gap, predictive of intramembranous ossification, and patterns of mild compressive stress in the periphery, consistent with endochondral ossification. These data derived from finite element analysis were consistent with previous histological findings.

Great interest surrounds research characterizing how mechanical forces may be translated into molecular signals that promote bone regeneration. Tong et al. described the role of focal adhesion kinase (FAK), a regulator of the integrin-mediated signal transduction cascade, in distraction osteogenesis [12]. In a rat model of mandibular distraction osteogenesis, immunolocalization of FAK in regions of new bone formation secondary to distraction was observed but was absent in the control groups where new bone formation occurred without distraction [12]. Similarly, investigations have also colocalized c-SRC, a kinase involved with activation of the mechanical transduction complex (p130), in regions of bone regeneration secondary to distraction osteogenesis [13]. In addition, microarray data from a rat model of distraction osteogenesis showed that FAK expression increases in response to mechanical stimulus [14]. While signaling molecules involved with transduction of mechanical forces are being identified, further work elucidating the mechanisms of these messenger molecules is required to clarify the influence of the mechanical environment on skeletal tissue engineering.

More recently, Ransom et al. have developed a mouse model for mandibular distraction, allowing for more detailed interrogation of the cellular and molecular events during guided bone regeneration [15]. Importantly, a localized clonal expansion of skeletal progenitor cells was identified leading to robust bone formation across the distraction gap. Furthermore, utilizing new techniques to investigate changes to the transcriptome and chromatin landscape, FAK-dependent mechanotransduction was found to activate a distinct regenerative program within a skeletal stem cell (SSC) population indicative of reversion to a more primitive neural crest genetic profile and different from transcriptional pathways activated by normal fracture healing. Inhibition of FAK abolished new bone formation, highlighting the importance of this mechanotransduction pathway in the activation of a gene-regulatory program and long interspersed nuclear element retrotransposons normally active in primitive neural crest cells. This reversion to a developmental neural-crest-like state by SSCs has been thought to underlie the robust bone tissue growth that facilitates stem-cell-based regeneration of the adult mandible with distraction (Fig. 55.1). These findings have thus expanded our understanding of how localized stem cell populations interact with their niche and have revolutionized our ability to potentially govern and regulate tissue healing and postnatal bone regeneration.

Distraction osteogenesis has also emphasized the multitude of growth factors that participate in bone regeneration. Elucidation of their roles and mechanisms will be a key element in successful tissue engineering. Transforming growth factor β-1 (TGF-β1), a potent growth factor known to stimulate osteoblast proliferation, has been demonstrated to have increased expression after osteotomy [16]. Immunohistochemistry revealed that TGF-β1 is localized to osteoblasts, primitive mesenchymal cells, and the extracellular matrix during the active distraction phase.

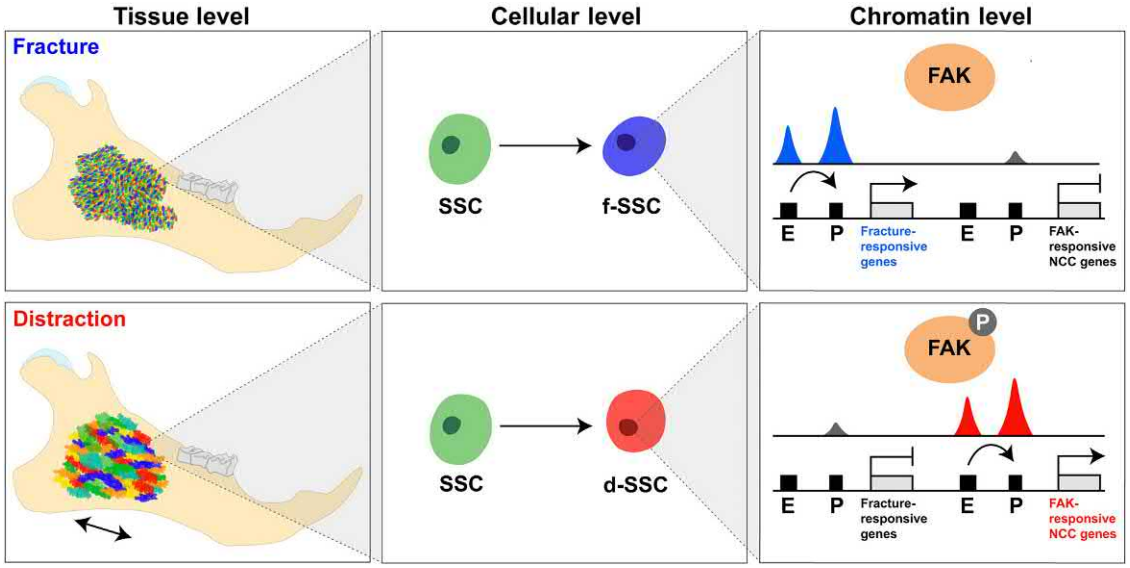

FIGURE 55.1 A comparison of the regenerative repair following fracture and MDO. At the tissue level: fracture results in a disorganized and nondirectional clonal proliferation that is diminished in magnitude (*left, top*). Bone lengthening following MDO, in contrast, results in expansion of large clones in a highly linear and directional manner (*left, bottom*). At the cellular level: the SSCs activated by fracture (f-SSC) (*middle, top*) retain postnatal SSC characteristics, whereas those activated by MDO (d-SSC) (*middle, bottom*) demonstrate plasticity and take on a NCC-derived signature. At the chromatin level: fracture promotes transcription of fracture-responsive genes in f-SSCs (*right, top*). In contrast, FAK-P in d-SSCs, there is a gain of accessibility at promoters (p) of FAK-responsive NCC-specific craniofacial and enhancers (E) (*right, bottom*). *FAK*, Focal adhesion kinase; *FAK-P*, phosphorylated FAK; *MDO*, mandibular distraction osteogenesis; *NCC*, neural crest cell; *SSC*, skeletal stem cell. *Adapted from Ransom RC, et al. Mechanoresponsive stem cells acquire neural crest fate in jaw regeneration. Nature 2018;563:514–21. doi:10.1038/s41586-018-0650-9.*

BMPs, another member of the TGF-β superfamily and known mediators in bone formation, have also been localized to regions of successful distraction osteogenesis. In examining femoral lengthening in rats, gene expression levels of BMP-2 and -4 were found to be elevated during the period of distraction [17]. Likewise, in distraction osteogenesis of rabbit tibia, immunohistochemical staining revealed intense BMP-2, -4, and -7 staining in fibroblast-like cells and chondrocytes during the distraction phase [18]. This was confirmed in membranous bone, where analysis of the bone regenerated from mandibular lengthening in rabbits revealed BMP-2 and -4 to be highly expressed in osteoblasts during distraction and in chondrocytes during consolidation [19]. Repeated local application of BMP-2 during distraction osteogenesis in a sheep model has been shown to significantly enhance the rigidity and architecture of the callus, indicating that enhancement of endogenous mechanisms may improve outcomes with this procedure [20]. Finally, transgenic mice have been used to show that the supporting vascular tissue, in particular smooth muscle and vascular endothelial cells, may be a significant source of BMP-2 during distraction osteogenesis [20]. Conversely, mesenchymal cells have been found to express proangiogenic growth factors within the distraction gap, suggesting that both bone and vascular tissue formation may be coordinated via a mutually supporting set of paracrine loops [21].

Insulin-like growth factor-1 (IGF-1) has also been implicated to play a role in the early stage of distraction osteogenesis. IGF-1 has been shown both in vitro and in vivo to stimulate osteoprogenitor cell mitosis and differentiation. In a canine tibia lengthening model, serum levels of IGF-1 increased initially during the early distraction period, followed by elevations in local IGF-1 at the region of distraction [22]. Schumacher et al. also presented evidence supporting the role of IGF-1 in early distraction, where periosteal IGF-1 levels in the rat tibia were increased only during active lengthening [23]. Exogenous addition of IGF-1 in a rabbit model of mandible distraction osteogenesis resulted in increased bone formation compared to control groups [24].

Lastly, studies have shown fibroblast growth factor (FGF) to be involved in promoting bone formation in distraction osteogenesis. Immunohistochemical analysis of sheep mandibles revealed FGF-2 staining in the region of distraction, with greater staining in animals found during high-rate distraction [25]. Exogenous administration of recombinant human FGF-2 (rhFGF-2) to a rabbit model of callus distraction following submetaphyseal corticotomy was also shown to increase the mineral content of the callus [26]. Further supporting the role of FGFs in bone formation, recent work utilizing a transgenic mouse with a gain-of-function FGF-receptor 3 mutation has shown accelerated ossification with distraction osteogenesis [27].

Distraction osteogenesis is thus a useful modality for translating discrete applications of mechanical force into molecular signals that induce skeletal regeneration. This process is not without its own morbidities however, such as soft-tissue infection, osteomyelitis, pin-tract infection or loosening, and patient discomfort [28]. Distraction osteogenesis is also limited by the need for complex hardware to accomplish distraction, and by the length of time required for large defects. Given these considerations, work continues on the development of alternative cell-based bone tissue engineering strategies to address large osseous defects.

Critical-sized defects

In the search for optimal elements of successful skeletal tissue engineering, the critical-sized defect has proven to be an indispensable tool for evaluating the performance of various constructs in the in vivo context. Critical-sized defects are bone defects that fail to heal without intervention, whereas noncritical defects may heal without planned reconstruction [29]. Critical-sized defects provide an easily accessible and quantifiable platform to evaluate the performance, dosages, and combinations of various cellular sources, osteogenic cytokines, and scaffolds.

Well-described bone defect assays include calvarial intramembranous bone, endochondral long bone, and segmental mandible defects [30]. The calvarial model is particularly useful in evaluating constructs for craniofacial defects given the relative ease in handling of the calvarial plate and support provided by the surrounding intact bone, absolving the need for fixation [31]. As it bears minimal mechanical load, the influence of exogenous forces on the investigation of specific cellular elements is reduced. Long bone segmental defects can also be created in the radius, where the ulna provides endogenous stability of that limb. Alternatively, in situations where load bearing of the construct is desired, femur defects can be used.

All these models are accessible to serial radiographic examination of the amount and density of mineralization using modalities such as plain films and micro-computed tomography. Histomorphometric analysis, however, allows cellular-level resolution and evaluation of bone resorption, changes in endogenous structure, and quality of the bone regenerate [30]. Serving as functional in vivo bone regeneration assays, critical-sized defects allow researchers to assess the rate and degree of bone healing for individual components of a bone construct and the combined effects of multi-modality treatments.

Cellular therapy

Over the past two decades, significant progress has been made in the identification of suitable cellular building blocks in regenerative strategies for bone engineering. Much of this has been due to an improved understanding in postnatal progenitor cells and fundamental bone biology. Resident progenitor cells can be found in many adult tissues and are active in endogenous mechanisms of repair and regeneration. Isolation, characterization, and utilization of these cells have provided many new promising treatment options. From a more developmental standpoint, studies have also begun to elucidate mechanisms involved in osteogenic lineage commitment of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) and a growing body of work has shown progress toward harnessing their differentiation potential for regenerative therapies [32,33].

Early studies on postnatal stem cells have identified resident progenitor cells in a wide range of tissues, including bone marrow, periosteum, muscle, fat, brain, skin, and umbilical cord blood [30], though bone marrow represents the most well-studied source. Over the years, substantial work has been performed to characterize the ability for bone marrow mesenchymal stromal cells (BMSCs) to be guided along multiple mesenchymal lineages [34] and BMSCs have been successfully applied across multiple animal models for skeletal tissue engineering. Both Ohgushi et al. and Bruder et al. documented the ability for implanted rat and human BMSCs, respectively, loaded onto calcium phosphate ceramics to accelerate healing and promote union across rat femoral defects [35,36]. Furthermore, in a rabbit calvarial model, BMSCs delivered on a fibrin scaffold demonstrated osseous regeneration similar to control implants seeded with osteoblasts [37]. Finally, multiple mouse models have revealed that both human and murine BMSCs may effectively contribute to bone formation in vivo [38–43].

An alternative mesenchymal cell population composed of adipose-derived stromal cells (ASCs) has similarly been isolated from postnatal fat and studies have likewise demonstrated the ability of these cells to form various mesenchymal components [44]. A frequently cited advantage for these cells is their ability to be procured in large numbers from individuals with minimal morbidity and used in an autologous fashion for tissue regeneration or reconstruction [45]. Lee et al. first demonstrated the in vivo ability of ASCs to generate bone by implanting polylactic-co-glycolic acid (PLGA) scaffolds seeded with predifferentiated rat ASCs subcutaneously [46]. ASCs have also been shown to be capable of healing osseous defects, as reported by Cowan et al. who used these cells to repair critical-sized calvarial defects in mice [39]. Significant bone formation could be appreciated at 2 weeks, with complete bony bridging by 12 weeks, and comparison of defects implanted with ASCs or BMSCs demonstrated similar rates of bone formation. These experiments established an important proof of principle

that ASCs could contribute to bone formation in a critically sized, skeletal defect in mice. Subsequent studies have since documented human ASCs to also be capable of stimulating bone regeneration in critical-sized defects in both rat and mouse models [47,48].

A third, well-studied source of adult tissue-resident progenitor cells for bone regeneration is skeletal muscle, from which muscle-derived stem cells (MDSCs) may be obtained [49]. Alone or in combination with BMP, MDSCs have been shown to improve bone regeneration of critical-sized calvarial defects in mice [50,51]. Recently, using the same mouse model, MDSCs were also described to have the ability to regenerate vascularized corticocancellous bone with a diploic space [52]. However, given the potential for MDSCs to form both osteogenic tissue and hematopoietic supportive stroma, it is possible that they possess distinct subpopulations of lineage-restricted progenitors. Furthermore, the in vivo osteogenic capacity of MDSCs may be dependent on BMP signaling [51], and MDSCs may have the same issues as BMSCs when it comes to donor site morbidity and a lower potential yield [53], limiting their utility in cellular-based regenerative strategies.

Despite the promise for application of these mesenchymal progenitor cells in regenerative medicine, however, there remains a lack of common standards and precise definition of cell populations that represents a major obstacle for research and utilization in skeletal tissue engineering strategies. The known heterogeneity of starting populations has made interpretation of results between different reports difficult, and extensive work has thus been devoted to evaluation of phenotypic surface antigen markers which, when used alone or in combination, may be employed to prospectively isolate more homogeneous fractions for bone regenerative applications [54]. Among BMSCs, studies have described use of Leptin Receptor that identifies a postnatal population of cells that proliferate after skeletal injury and possess the ability to differentiate into bone and adipose tissue [55]. Nestin expression may also be used to isolate a population of self-renewing mesenchymal cells within bone marrow with enhanced osteogenic differentiation capabilities [56] and Gremlin has been reported to identify a population of osteochondrorecticular cells located within the metaphysis with the capability to differentiate into bone, cartilage, and reticular marrow stromal cells [57]. Finally, studies have shown use of platelet-derived growth factor (PDGF) receptor α and Sca-1 to be capable of isolating a perivascular population of progenitor cells from bone marrow with the capacity to differentiate into hematopoietic, osteogenic, and adipogenic tissues [58].

In similar fashion, many studies have been published describing use of various surface antigens in ASCs to prospectively identify subpopulations with increased osteogenic capacity. An enriched fraction low in CD105 (endoglin) expression has been shown to possess enhanced in vitro osteogenic differentiation and the ability to promote faster healing of critical-sized calvarial defects in mice compared to an unenriched population [59]. This has been likewise demonstrated with CD90 (Thy1), where positive selection for cells with this surface maker has been found to identify a subpopulation more capable of both in vitro and in vivo bone formation [60]. And as BMPs have been shown to accelerate osteogenic differentiation, prospectively isolating ASCs with increased BMP receptor-IB expression has been reported to delineate a highly osteogenic fraction of cells for potential use in bone regenerative strategies [61].

However, despite our expanding knowledge with the use of specific cell-surface proteins to improve the enrichment of mesenchymal progenitor cells for skeletogenic activity, cells prospectively isolated with these markers are still nonetheless incredibly heterogeneous. And what has been previously described as "mesenchymal stem cells" based on their adherence to plastic culture plates likely represents a population comprising multiple distinct lineage-committed progenitors rather than a uniformly purified bone forming stem cell. Only recently has a more precise functional hierarchy of skeletal tissue-forming cells been defined. The existence of a true multipotent SSC was first demonstrated by Chan et al. who, with the use of rainbow-reporter mice, identified clonal expansion of bone at growth plates encompassing only bone, cartilage, and stromal tissue [62]. This suggested that these tissues arise from lineage-restricted stem and progenitor cells that do not also give rise to hematopoietic, adipose, or muscle tissue. Subsequent work has defined a lineage tree of skeletal stem/progenitor cells with the mouse SSC giving rise to a bone, cartilage, stromal progenitor (BCSP) and other downstream committed progenitors with the capacity to specifically form cartilage, bone, or stromal tissue (Fig. 55.2). Interestingly, subpopulations within the skeletogenic tree have been found to encompass mesenchymal subtypes previously identified by prospective isolation strategies. Both Leptin receptor and Nestin-expressing cells have been shown to overlap with the mouse SSC population [63].

Importantly, identification of the mouse SSC has substantially deepened our understanding of bone and cartilage repair and regeneration. Growth factors such as BMPs have been extensively used to successfully promote bone formation, though overactive signaling has also been linked to pathologic heterotopic ossification. Studies in mice have revealed that BMP-2 can induce skeletal reprograming through de novo formation of SSCs, even within extraskeletal sites such as adipose tissue [62]. Emerging evidence also suggests that blocking vascular endothelial growth factor (VEGF)-dependent ossification can drive chondrogenic differentiation of SSCs. Following skeletal

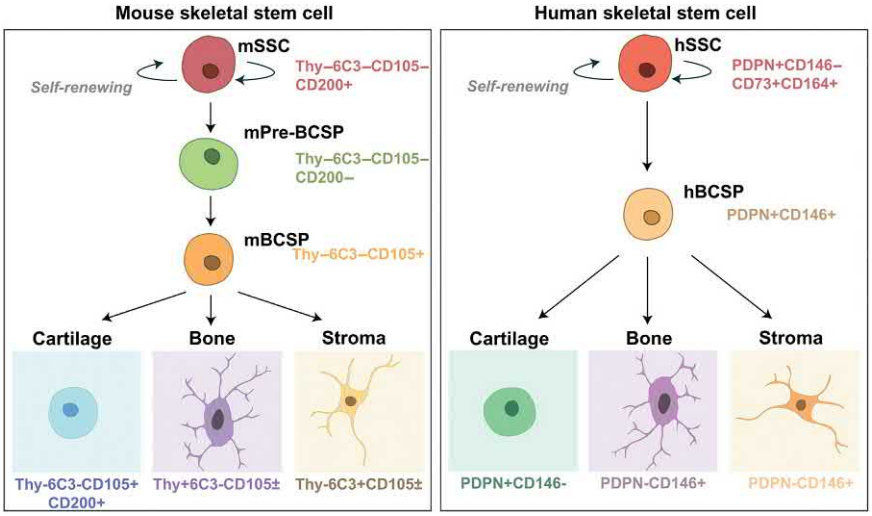

FIGURE 55.2 Schematic representation of the mouse (left) and human (right) SSC lineage trees. The surface markers used to isolate the distinct cell types by flow cytometry are shown. All cells in the mouse SSC tree are negative for the mouse lineage markers (CD45, TER119, Tie2) and positive for AlphaV. All cells in the human SSC tree are negative for the human lineage markers (CD45, CD235a, CD31, Tie2). *BCSP*, Bone cartilage stromal progenitor; *SSC*, skeletal stem cell. *Adapted from Chan CKF, et al. Identification of the human skeletal stem cell. Cell 2018;175:43–56.e21. doi:10.1016/j.cell.2018.07.029.*

injury, expansion of a specific fracture-induced BCSP (f-BCSP) downstream of the SSC has also been identified with enhanced skeletogenic potential secondary to gene expression patterns recapitulating perinatal skeletogenesis [64]. In the setting of diabetes, where fracture repair may be impaired due to suppression of SSC expansion, pharmacologic strategies to restore SSC proliferation and osteogenic potential has been shown to rescue bone healing in a diabetic mouse model [65]. These reports point to the future development of molecular strategies aimed toward the modification of specific progenitor cell microenvironments to address skeletal deficiencies, underscoring the impact of the SSC discovery in mice.

Recent studies have now extended findings to the human skeleton, where a similar SSC and hierarchical tree have been described [66]. By using a mouse SSC/BCSP-specific human ortholog gene set, human SSCs have been identified in the prehypertrophic and hypertrophic zones of fetal femoral growth plates. However, with divergence in surface protein expression between mouse and human cells, flow-cytometric analysis has revealed a distinct panel of surface markers characterizing the human SSC (Fig. 55.2). Nonetheless, these cells are still capable of lineal generation of chondrogenic and osteogenic subsets and reside at the top of the skeletogenic differentiation tree like their mouse counterpart. Studies with human fetal phalanges engrafted into the flank of immunodeficient mice have similarly shown fracture-induced amplification of human SSCs in response to skeletal injury, and BMP-2 has likewise been found to induce SSC expansion at extraskeletal sites, including freshly isolated human adipose tissue. Understanding how these cells can be controlled to regenerate bone may thus allow for alleviation of some of the burden of chronic musculoskeletal disorders associated with aging and disease. Perhaps the greatest implication of this work, however, relates to our comprehension of convergent and divergent skeletal regulatory programs between mouse and human bone progenitors. Assays characterizing differences in transcriptomic and genomic landscape between mouse and human SSCs have defined distinct features in accessibility regions reflective of species-specific gene expression patterns that may be the result of divergent evolution in epigenetic regulation. These discoveries may have direct significance in our design of bone regenerative strategies and interpretation of translational results.

In contrast to the more skeletal lineage-restricted potential of postnatal progenitor cells, work has also been done with pluripotent stem cells for bone tissue engineering. Despite the broad differentiation potential of ESCs, ethical and political controversies have fueled pluripotent cell research toward adult-derived iPSCs for tissue engineering [67]. In vitro studies have demonstrated osteogenic gene expression and bone forming capabilities of mouse ESCs and iPSCs to be similar, making iPSCs a preferred source of osteogenic cells [68]. While iPSCs have the capacity to spontaneously differentiate across all three primary germ layers, researchers have developed methods to drive differentiation of iPSCs into more pure populations of a single lineage for developmental research, drug screening, disease modeling, and for cell and tissue replacement. However, barriers to the use of iPSC technology for clinical applications include availability of a large autologous cell source, efficiency and safety of reprograming, defined optimal culture conditions, efficiency of directed differentiation, tumorigenicity risk, and stability and immunogenicity of transplanted cells. Nonetheless, significant progress has been made in addressing some of these clinical hurdles for use of iPSCs in bone regenerative strategies.

A number of adult tissues, from skeletal muscle satellite cells to circulating T cells, can serve as a source of

patient-specific cells for iPSC reprograming [69,70]. Notable for reconstructive surgeons, fibroblasts from skin biopsies and ASCs from liposuction aspirate provide readily available, large quantity cell sources that can readily yield iPSCs [71]. Initial reprograming experiments used lentiviruses expressing cancer-related genes c-Myc and Klf4, and transcription factors Oct4 and Sox2, introducing risk of insertional mutagenesis at the sites of viral integration and downstream tumorigenic risk from continued oncogene expression [72]. A variety of reprograming methods have subsequently improved on lentiviral technology using nonintegrating minicircle plasmids [73], microRNA [74], stabilized mRNA [75], and nucleotide-free recombinant protein [76]. Though these methods result in lower efficiency of reprograming than the lentivirus, they carry a reduced risk of mutagenesis and have better safety profiles.

Important to engineering of skeletal bone, iPSCs have been demonstrated to respond robustly to osteogenic inductive media containing β-glyerophosphate and ascorbic acid to form osteoblasts in vitro [77]. Following induction of differentiation with embryoid body formation, addition of these components has been shown to drive in vitro osteogenic differentiation of mouse iPSCs, as demonstrated by upregulated *RUNX2* and *Col1a1* gene expression and histologic staining for extracellular matrix mineralization. Furthermore, addition of TGF-β1 increases bone nodule formation after treatment of iPSCs with osteogenic media [78]. For use of iPSCs in vivo, cells must be differentiated sufficiently to avoid uncontrolled and undesired proliferation leading to teratoma formation. For this reason, many studies have first derived mesenchymal cells from mouse iPSCs prior to successful use in the reconstruction of mouse critical-sized calvarial defects [79]. Alternatively, transgenic mouse iPSCs expressing SATB2, a gene critical in normal bone development, transplanted directly into the same defect site has been reported to promote calvarial bone regeneration without teratoma formation [80]. Human iPSCs have also been used to generate mesenchymal cells that have then been shown to successfully repair critical-sized rat calvarial defects [81]. Finally, the calvarial niche is naturally composed of high concentrations of cytokines and bone progenitors and manipulation of this microenvironment may be potentially used to drive in situ osteogenic lineage commitment by transplanted iPSCs. In support of this, Levi et al. found that delivery of undifferentiated human iPSCs on a biomimetic scaffold, in conjunction with osteogenic factors such as BMP-2, was sufficient to both strongly drive osteogenic differentiation and dramatically reduce the incidence of teratoma formation [82].

Research granting agencies, both private and public, have dedicated significant funds to advancing the translation of stem cell applications toward clinical trials and therapeutic development. Continued work to advance transplantation of tissue-specific progenitors such as BMSCs, ASCs, MDSCs, and SSCs, or pluripotent cells such as ESCs and iPSCs toward the actualization of novel therapies will need to address challenges of immune privilege and rejection, the establishment of standard operating protocols for defined culture and storage conditions, tailoring cell/scaffold combinations for specialized craniofacial regions, and defined predictability of graft retention and integration into host. As these are active areas of research undergoing continuous rapid advancements, the field continues to hold promise for future skeletal reconstructive applications.

Cytokines

Studies from developmental biology and distraction osteogenesis have revealed a host of growth factors involved in cell proliferation, differentiation, adhesion, and migration during bone formation. An understanding of these interactions will undoubtedly allow for their use in bone tissue engineering. Successful skeletal tissue engineering will likely involve the incorporation of or stimulation by appropriate cytokines combined with the delivery or recruitment of a bone forming progenitor population. Some examples of the most well-studied cytokines include BMPs, IGFs, FGFs, PDGFs, and VEGFs [83].

Already in use clinically, BMPs hold the most promise as a growth factor for use in skeletal tissue engineering. BMPs were originally isolated by Urist from bovine bone extracts and were found to induce ectopic bone formation subcutaneously in rats [84]. This large group of proteins, comprising nearly one-third of the TGF-β proteins, has also been found to be involved in mesoderm induction, skeletal patterning, and limb development. BMPs are known to control both intramembranous as well as endochondral ossification through chemotaxis and mitosis of mesenchymal progenitor cells, induce lineage commitment to osteogenic or chondrogenic pathways, and regulate programed cell death [85]. BMPs transmit their signals via ligand binding to the heteromeric complex of type I and II serine/threonine kinase receptors on the cell surface. The ligand signal is then transduced intracellularly via activation of SMAD (signaling mothers against decapentaplegic) proteins, which subsequently migrate to the nucleus to effect gene expression. Noncanonical BMP signaling has also been shown to occur through the MAP-kinase (mitogen-activated protein) pathway [86].

BMP-2, -4, -6, -7, and -9 have been well studied most as osteoinductive agents. It is thought that BMPs regulate osteoblast differentiation via increased transcription of core-binding factor-1/Runt related family 2 (Cbfa1/Runx2), a molecule known to be necessary for commitment along an osteoblastic lineage [87]. These osteoinductive BMPs

have been demonstrated to promote osteogenic differentiation in multiple cell types, including fibroblasts, chondrocytes, BMSCs, and ASCs, and as previously discussed, BMPs have also been shown to induce formation of SSCs [62]. The effect of BMPs has also been noted to be concentration dependent; at low concentrations, they foster chemotaxis and cellular proliferation, but at high concentrations, BMPs induce bone formation [88]. Interestingly, in culture and in vivo, BMP-2/6, -2/7, and -4/7 heterodimers have been shown to more effectively enhance osteogenesis than homodimer combinations [89,90]. Recent work has now focused on delivery of BMP-2 in sustained fashion to more effectively stimulate bone formation. Keeney et al. found that integration of minicircle DNA incorporating a BMP-2 transgene into PLGA scaffolds could effectively sustain BMP-2 release by transfected cells and enhance calvarial reconstruction in a mouse model [91]. And as previously discussed, BMP-2, in concert with VEGF inhibition, may be capable of promoting cartilage formation [62]. Importantly, BMP-2 has been approved for use by the FDA for surgical reconstruction of various bone defects, and though some controversy exists over clinical outcomes and concerns have been raised over ectopic bone formation and inflammation, most studies show that treatment with BMP-2 has promising clinical efficacy.

IGF-1 and IGF-2 are 7.6 kD polypeptides that have been demonstrated to stimulate bone collagen synthesis, as well as osteogenesis and chondrogenesis. A transgenic mouse with upregulated IGF expression in osteoblasts was found to have increased bone formation of the distal femur as compared to control mice [92]. Of interest, however, histology did not reveal an increase in the number of osteoblasts, suggesting that IGF-1 increased the activity of existing bone progenitor cells. Conversely, in an IGF-1-null transgenic mouse model, the size and bone formation rates of the knockout mice were significantly reduced as compared to their wild-type littermates [93]. From a skeletal tissue engineering perspective, studies have suggested age-related reduction in IGF-1 production to be associated with impaired bone forming capacity of human mesenchymal progenitor cells derived from elderly patients, and overexpression of IGF-1 can enhance biomineralization of these cells in culture [94]. IGF-1 was also shown to enhance the ability of bone marrow-derived mesenchymal progenitor cells to restore osteochondral defects in rabbit knee joints [95].

FGFs consist of a highly conserved family of 24 known peptides that transmit their signals via a family of four transmembrane tyrosine kinases. FGF-2, the most abundant ligand of the family, is known to increase osteoblast proliferation and enhance bone formation in vitro and in vivo [96]. In FGF-2 haplo-insufficient and null mice, decreased bone mineral density has been observed, and impaired bone formation has also been correlated with decreased expression of FGF receptor-2 [97]. Application of exogenous FGF-2 has been shown to rescue bone nodule formation in osteoblast cultures from these FGF-2 mutant mice. More recently, utilizing a beagle dog periodontal defect model, exogenous FGF-2 with a hydroxypropyl cellulose vehicle was shown to increase angiogenesis and osteogenesis in vivo by increasing local expression of BMP-2 and other osteoblast differentiation markers within the regenerate [98].

Platelet-derived growth factor, a 30 kD polypeptide, has also been demonstrated to be a potent stimulus for osteoblast proliferation, chemotaxis, as well as collagen activity. In particular, PDGF-BB has been shown to upregulate osteogenic gene expression and extracellular matrix mineralization by ASCs [99]. The utility of PDGF in vivo has been shown with enhanced bone formation after local application to tibial osteotomies in rabbits and canine mandibles after periodontal surgery. PDGF, also studied for chronic wounds, is now being evaluated clinically for periodontic indications. A recent multicenter trial demonstrated that the application of recombinant human PDGF in a tricalcium phosphate matrix resulted in significantly increased periodontal bone formation [100].

Finally, the recruitment of blood vessels has long been known to be vital to support newly formed tissue. In support of this, application of VEGF to both intramembranous and endochondral bone defects resulted in increased blood vessel formation, ossification, and new bone in various mouse and rabbit models [101]. Not only does VEGF have a role in angiogenesis, but it may also stimulate osteoblast differentiation and osteoclast activation for bone formation and remodeling. Inhibition of VEGF by application of a receptor neutralizing agent has also been shown to diminish blood vessel formation and osteogenesis [102].

Interestingly, a direct comparison of the ability of VEGF-A, BMP-2, and FGF-2 to enhance intramembranous bone healing in a critical calvarial defect has revealed that application of each of these cytokines increases proliferation of osteoblasts and progenitors in the defect region [103]. While all three cytokines significantly enhanced healing of the defect, VEGF-A and BMP-2 increased angiogenic support, while the effects of FGF-2 were independent of new blood vessel formation. Supporting the hypothesis that angiogenesis is critical to calvarial bone healing, VEGF-A and BMP-2 applications also resulted in the most bone formation, with nearly complete healing in a mouse critical-sized defect at 3 weeks [103].

Scaffolds

The challenges of incorporating cytokines in skeletal tissue engineering involve identifying cell populations and molecules that stimulate and participate in bone formation, but

the effectiveness of these treatments is largely based on the mode of delivery, effective dosages, and compatible carrier mechanisms. Fortunately, advances in materials science have provided an abundance of innovations for developing an appropriate carrier for these cells and molecules. In the selection of a biomimetic scaffold for engineering bone, the material should be osteoconductive, osteoinductive, biocompatible, and biodegradable without eliciting an immune response. Osteoconductivity refers to the ability of the graft to support the attachment of cells and allow for new cell migration and vessel formation. The osteoinductive quality of scaffolds describes their ability to guide progenitor cells along an osteogenic lineage. Some of the challenges in designing the structure of scaffolds lie in the need to maximize the porosity of the scaffold to promote cellular and neovascular ingrowth while maintaining the structural integrity of the lattice [30]. Potential matrices can be generalized into three categories: natural, mineral-based, and synthetic polymers.

Natural scaffolds are typically biodegradable and include the use of collagen, hyaluronic acids, calcium alginate, and chitosan. In many instances, they exhibit osteoinductive properties, exclusive of cells or cytokines. Implantation of collagen type I alone into critical-sized defects of rat mandibles resulted in partial bone healing [104]. However, one disadvantage of natural scaffolds is their lack of mechanical stability, hence, their limited utility for use in load-bearing regions of the skeleton. Clinical application of natural scaffolds in patients is also limited by the biochemical changes often induced by sterilization procedures. Some of these limitations, in particular structural integrity, can be offset by using composites that combine the best properties natural scaffolds with other materials discussed below [105].

Mineral-based scaffolds include calcium phosphate ceramics and bioactive glass. Calcium phosphate ceramics are available as HA or β-tricalcium phosphate, with HA most closely mimicking the structural and chemical characteristics of the mineral component of bone. Tricalcium phosphate is marked by a high-dissolution rate that accelerates material resorption and elicits an immune response, while HA has high chemical stability. Mineral-based scaffolds provide an osteoinductive signal to encourage differentiation of progenitor cells along an osteogenic lineage. There is, however, great variability in the quality of calcium phosphate ceramics to support osteogenesis due to the difficulty in reproducibly creating these scaffolds [106], and many formulations have been found to be too brittle alone for use in skeletal reconstruction of load-bearing regions.

Synthetic polymer scaffolds, including polylactic acid (PLA), polyglycolic acid, polydoxanonone, and polycaprolactone (PCL), have been engineered to provide a greater ability to withstand mechanical forces. These lattices are marked by their incredible strength while also being designed to be biodegradable via hydrolysis. Polymer scaffolds allow ingrowth of bony tissue but lack osteoinductive properties. To overcome this deficiency, polymer scaffolds, such as PLGA, have been combined with mineral coating to create an osteoconductive niche with enhanced architectural strength. HA-coated PLGA scaffolds have been extensively used in various animal models to promote bone reconstruction [39,107,108].

Hydrogels represent another class of polymer scaffolds that are formed by polymerization and cross-linking of molecules such as collagen, acrylic acid, and *N*-isopropylacrylamide [109]. Hydrogels are an attractive option because of their temperature-dependent physical properties. Synthetic formulations can be designed to be gelatinous at room temperature but take on more rigid qualities at body temperatures. This property allows for the administration of tissue-engineering constructs via injection. Hydrogels also allow for relatively easy chemical manipulation of individual peptides, making them bioavailable as free molecules in the matrix or tethered for sustained release. Incorporation of arginine−glycine−asparagine (RGD) peptide motifs on these polymers has been demonstrated to enhance osteoblast adhesion and proliferation [110]. By combining collagen hydrogels with PLGA microparticles to deliver osteogenic mesenchymal progenitor cells, researchers have described a cell delivery mechanism that is elastic enough to adapt to the shape of a defect but will mineralize and acquire structural rigidity in the presence of differentiating cells [111]. Other groups have used a hybrid scaffold by combining an osteoconductive load-bearing polymer matrix with a peptide hydrogel providing controlled release of recombinant human BMP (rhBMP)-2 [112]. Cells seeded on this scaffold demonstrated increased expression of in vitro bone differentiation markers. However, progress over the last several years with nanoengineering has now shown promise for incorporation of two-dimensional nanosilicates in nanoengineered hydrogels to eliminate this need for BMP-2 [113]. Hybrid hydrogels may thus provide for multiple reconstructive goals, including structural integrity, sustained release of cytokines, and support of cell differentiation, and are one of the more promising scaffold candidates for actualizing large defect reconstruction.

Lastly, electrospinning has emerged in recent years as a novel method to construct scaffolds that closely mimic natural extracellular matrix. Nanowoven fibers are generated by shooting a jet of polymer solution through a high electric field [114]. Adjusting the polymer solution and the electric field allows the fiber diameter and porosity to be controlled with high precision. By blending various materials using this technique, structural properties of the resultant scaffold can be tightly controlled. For example, electrospun PCL/PLA nanofibrous scaffolds have been

found to improve human mesenchymal progenitor cell osteogenesis and lead to robust bone formation in vivo [115]. Silk fibroin has also been incorporated into three-dimensional electrospun structures for bone tissue engineering purposes [116].

Of note, many different osteogenic materials or cytokines can also be incorporated into electrospun scaffolds, accurately reproducing natural bone matrix with embedded osteoinductive factors. Along these lines, silk fibroin fiber scaffolds incorporating BMP-2 and nanoparticles of HA have been shown to enhance mineralization of human bone marrow mesenchymal cells cultured on these scaffolds in vitro [114]. Electrospun biodegradable fibers seeded with amniotic-derived mesenchymal progenitor cells were likewise used to generate allogeneic fetal bone grafts, which were superior for in vivo reconstruction of craniofacial defects compared to prosthetic materials in a rabbit model [117].

Tissue engineering in practice

Clinical applications for tissue engineering have long focused on the delivery of BMP cytokines and use of cytokine-scaffold devices. More recently, however, alternative strategies have been reported with use of other cytokines or cell-based approaches. Nonetheless, with many of these applications, criticism has still been voiced over the need for high doses of cytokines necessary to obtain clinical relevance and the resulting costs associated with manufacturing growth factors for patient use. Concern also exists about rare but reported incidences of high blood pressure and even myocardial infarctions in animal models, which may be secondary to an immunologic response to implanted constructs [118].

The Infuse Bone Graft/LT Cage fusion device has been used in over one million procedures worldwide and is currently approved by the FDA for use in spinal fusion, tibia fractures, and oral–maxillofacial reconstruction of bone defects associated with teeth extraction (Medtronic Sofamor Danek, Minneapolis, MN). This product involves an absorbable, bovine collagen implant soaked with rhBMP-2. Importantly, humans require supraphysiologic doses ranging from 0.4 to 1.5 mg/mL of rhBMP-2 to form new bone, and because of these high doses, there have been some concerns raised about excess bone growth around the growth factor implantation site. Interestingly, a clinical study of Infuse in 74 consecutive patients revealed radiographic lumbar fusion in 100% at 12 and 24 months without any abnormal bone overgrowth. However, this study was significantly weakened by the lack of a control group [119]. Off-label use for other procedures in spine have been reported to cause complications including tissue swelling, seroma, ectopic bone formation, paralysis, and death [120,121]. This has led many surgeons to reconsider the broad use of Infuse for off-label applications. And given reports describing a link between osteosarcoma and BMP activity [122], clinicians have also been hesitant to use Infuse in the pediatric population. Of note, several recent animal studies have actually found BMP-2 to inhibit tumor expansion and development of lung metastases through induction of bone formation in heterogeneous osteosarcoma cells [123,124].

rhBMP-2 has also been used in patients requiring maxillary sinus floor augmentation, in preparation for endosseous dental implants. In one study, rhBMP-2 was applied to the maxillary sinus floor on an absorbable collagen sponge at 0.75 and 1.5 mg/mL [125]. Radiographic evaluation for bone mass in the maxillary sinus floor revealed that rhBMP-2 with collagen sponge alone was able to induce bone formation, but this was found to be less effective than autogenous bone grafting in fostering new bone formation. Notably, however, rates of successful placement of dental implants were similar between the bone graft treatment arm and patients receiving rhBMP-2 [125].

Similar to BMP-2, rhBMP-7, originally marketed under the brand name OP1, has been studied to aid in the fusion of vertebral bodies and to treat critical-sized bone fractures. It is also delivered via a bovine collagen matrix and has been used in limited situations to treat nonunion in the tibia of at least nine months, secondary to trauma, in skeletally mature patients. However, in a small case series report, the use of rhBMP-7 in five patients who had failed allografts did not result in any significant healing [126]. Compared to rhBMP-2, rhBMP-7 may also be less effective at promoting bone formation following spinal procedures and has thus struggled to obtain FDA approval [127].

In addition to BMPs, other cytokines have now emerged for use in human clinical trials to promote bone formation. In particular, a recent randomized double-blinded multicenter trial investigating the use of rhFGF-2 in combination with a β-tricalcium phosphate scaffold showed that there was significant improvement in linear bone growth and percentage of bone fill at vertical periodontal defects with use of this construct [128]. Akin to these findings, Kitamura et al. also observed the ability for rhFGF-2 within a 3% hydroxypropylcellulose delivery vehicle to stimulate regeneration of periodontal bone in patients without any serious adverse events noted throughout the clinical trial period [129].

Aside from clinical use of growth factors for skeletal tissue engineering, studies have also begun to evaluate cell-based strategies for bone regeneration in patients. For example, several reports have looked at the use of BMSCs for skeletal reconstruction. A wide range of studies in the literature have shown some promise for use of these cells in applications ranging from nonunion and fracture healing, to segmental bone defects and spinal

fusion, to distraction osteogenesis [130]. Of particular interest, a recent randomized, controlled clinical trial investigating the effect of bone regeneration in the maxillary sinus using autologous mesenchymal progenitors derived from bone marrow on a β-tricalcium phosphate scaffold revealed higher density of engineered bone compared to patients receiving scaffold alone [131]. Furthermore, the greatest benefit was observed in patients with the most severe alveolar deficiencies, with superior bone quality noted compared to control patients who did not receive autologous cells. These results were also correlated with degree of CD90 + progenitor cell enrichment, and the higher the CD90 composition of transplanted cells, the greater the regenerated bone. Not surprisingly, this parallels the previously discussed work by Chung et al. highlighting enhanced osteogenic potential of this same mesenchymal cell subpopulation [60].

Promising results are nonetheless tempered by other studies that have questioned the long-term stability of clinically reconstructed skeletal defects using mesenchymal cells. Thesleff et al. recently reviewed five patients who had undergone prior calvarial defect repair with ASCs, β-tricalcium phosphate granules, and titanium or resorbable mesh. While initial results were found to be promising and no serious complications were noted, long-term follow-up at 6 years revealed many patients with marked resorption bone and only two of the five showing stable, successfully ossified grafts [132]. However, substantial heterogeneity among the harvested cells used may have contributed to these suboptimal results, and continued efforts to refine our understanding of functionally distinct mesenchymal cell subpopulations, along with the recent discovery of the mouse and human SSC, lend confidence to the development of future successful cell-based strategies for skeletal tissue engineering.

Conclusion

The intersection of advances in stem cell biology, molecular biology, biochemistry, bioengineering, and materials science has brought to the forefront the ability of regenerative medicine to address problems of skeletal defects. Efforts are furthered by continued research on identification of postnatal osteoprogenitor cells, the functions and interactions of osteogenic cytokines, and scaffold design. In particular, the influence of nanotechnology on scaffold development and the advancements of growth factor releasing scaffolds and hydrogels are promising advancements that may help one to promote bone formation by progenitor cells without the need for supraphysiologic doses of cytokines such as BMP-2. Nonetheless, efforts are still needed to develop methods for creating vascular support of large osseous constructs, and this may potentially be achieved through the use of proangiogenic agents. Further investigation into identifying and enriching available osteoprogenitor populations now that the SSC and hierarchical tree have been described along with continued advancements in pluripotent cell research may lead to future human clinical trials and more available tools for reconstructive surgeons. Given the immense biomedical burden of skeletal defects and the significant developments in skeletal tissue engineering, osseous regeneration provides a promising and attainable goal that will be achieved through interdisciplinary collaboration.

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Part Fifteen

Nervous system

Chapter 56

Brain implants

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Introduction

Even though regulatory-approved cellular brain implant therapies are not yet available, experimental data strongly support the use of cellular brain implants and tissue engineering concepts in the treatment of brain disorders and promise new disease-modifying medical products for patients. Various cellular implants have already been applied in the clinic in proof-of-concept studies in Parkinson's disease (PD) [1−3], Huntington's disease (HD) [4−6], Alzheimer's disease (AD) [7−9], epilepsy [10], and stroke [11−13]. Due to its defined anatomy and relatively well-established disease mechanisms, PD is a major therapeutic target for cellular brain implants and is highlighted in this chapter as a disease example to illustrate the tissue engineering concepts applied to date.

Despite the long-standing success of many drugs for PD, such as L-dihydroxyphenylalanine (L-DOPA) therapy, the current treatments of PD do not stop the progressive dopamine neuron dysfunction and cell death. Over time, patients on chronic L-DOPA therapy develop both progressive symptoms and drug-induced side effects and require additional treatment options. For these patients so-called deep brain stimulation (DBS), which utilizes implantable neural stimulators that inhibit neuronal transmission in local areas of the brain, often the subthalamic nucleus, can yield good therapeutic results without inducing permanent lesions in the brain [14,15]. These devices are Food and Drug Administration approved and are commercialized by several companies, with Medtronic, Inc. being the most well-known. Despite successful applications of DBS for the treatment of PD, this therapy is based on the inhibition of normal neurons to compensate for the disease damage. DBS does not address the biology of the underlying disease itself and, albeit successfully applied in many patients, the compensatory destruction or inhibition of normal tissue is not an optimal treatment for neurological disorders. There is therefore a need for new treatment strategies that can address the pathology more directly and offer disease-modifying effects. Fortunately, the accumulated knowledge of the pathological processes, molecular and cell biology, biomaterials, imaging, and successful implementation of DBS and its associated stereotactic neurosurgical procedures make it now possible to implement disease-modifying brain implants based on tissue engineering concepts to the treatment of PD and other neurological diseases.

In many untreatable neurological disorders, the progressive loss of neurons and their associated function is the primary underlying cause for the symptoms of the disease. Therefore various cell implant strategies have been designed to either *replace* the neurons or their function or to *protect and/or regenerate* the function and health of the diseased neurons, or a combination of both (Fig. 56.1).

Clinical applications to replace the dopaminergic function in patients with PD have so far mainly utilized primary tissues or cells but more recently early clinical trials with stem cells are underway [2,16]. Dopaminergic neurons derived from stem cells and more sophisticated tissue-engineered implants may thus be developed to replace diseased and lost dopaminergic neurons and have a potential to cure PD [17]. This chapter reviews some of the cell replacement and regenerative brain implants applied in the clinic and touches on what may be developed in the future.

Cell replacement implants

Primary tissue implants

As mentioned earlier, oral L-DOPA therapy remains the main treatment for PD. L-DOPA is a precursor to dopamine that passes the blood−brain barrier and is mainly taken up by the residual dopaminergic neurons that convert L-DOPA to dopamine and increase their dopamine

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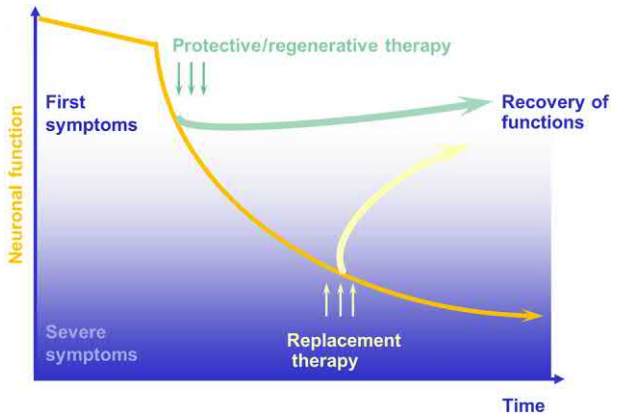

FIGURE 56.1 The concepts of cell replacement and protection/regeneration are depicted. A loss of neurons or their function (*orange line*) occurs with aging but in a disease, such as PD, neuronal loss is accelerated and after a while, the loss is significant enough to cause symptoms of the disease. At this stage, further deterioration could be prevented by protecting the neurons and the system could even be improved by applying regenerative factors to the diseased host cells. After significant loss of neurons, regeneration of the host system is not possible. However, the implantation of cells capable of replacing the function of host cells could be possible. Both approaches have been applied in PD and a combined treatment of neuroprotection/regeneration with replacement may be achieved in future brain implants. *PD*, Parkinson's disease.

production and storage. However, with the progressive loss of dopaminergic neurons, the L-DOPA therapy eventually becomes ineffective and severe fluctuations in the ability to initiate movements occur. Because L-DOPA can increase the production of dopamine and alleviate the symptoms of PD, a reasonable therapeutic approach may be to implant dopamine- or L-DOPA-secreting cells in the relevant areas of the brain (striatum).

Considering this idea, the first clinical transplantation for PD using a cellular brain implant was performed at the Karolinska Hospital in Stockholm, Sweden in the early 1980s [18]. Autologous dopamine–secreting adrenal chromaffin cells were harvested from one of the patient's adrenal glands and successfully transplanted to the striatum. The procedure was adopted very quickly by the medical community and initial reports indicated good clinical results. With time though, other studies showed poor survival of the cells and minimal positive clinical effects, resulting in the cessation of the treatment [19]. However, the concept of cellular brain implants had made its definite entry into the clinic and paved the way for cell therapies of the brain.

At about the same time as the first clinical chromaffin cell transplants were made at the Karolinska Institute/Hospital in Stockholm, preclinical research at the same institution demonstrated the concept of using fetal tissue dopamine grafts capable of replacing the loss of function of lesioned endogenous dopaminergic neurons [20]. This promising cell transplantation strategy for PD was developed further by Björklund and Lindvall at Lund University in southern Sweden [1]. After several years of extensive validation of the concept in animal models, cells were transplanted to the striatum of two PD patients [21]. The first results were safe but relatively unimpressive from a cell survival perspective, prompting modifications to various parts of the experimental procedure, and a second pair of patients transplanted about 1 year later fared much better [22]. These patients showed positive clinical recovery starting about 4 months after the procedure. Positron emission tomography (PET) data indicated that the grafts survived and took up and secreted dopamine. More than 10 years after the procedure, one of the patients showed persistent graft viability on PET scanning and required only minimal L-DOPA therapy [23,24]. To date, more than 300 patients have been transplanted with fetal ventral mesencephalic tissue at different centers around the world with mixed but encouraging results. However, ethical considerations, the lack of suitable donor material, the heterogeneity of the tissues and preparations, and the inability to industrialize the process, have all made it difficult to make standardized medical trials and therapy geared for a large number of patients. Two National Institutes of Health–sponsored controlled trials with fetal transplantation showed only minimal efficacy and some patients developed movement side effects (dyskinesias) that were related to the grafting procedure [25,26]. It has also been observed that grafted fetal neurons may be able to survive for many years after surgery, but these same grafted neurons are subject to the same pathological processes that underlie the loss of endogenous dopaminergic neurons in PD [27]. Therefore fetal transplantation as a therapy for PD is no longer actively pursued as a therapeutic option except on an experimental level and 11 patients were recently transplanted in a highly controlled trial aims to minimize variables identified in previous trials and establish protocols for future stem cell transplantations [16]. New stem cell replacement strategies for PD will therefore replace primary tissues and cells. However, the translational studies with chromaffin and fetal–derived primary tissues have generated important data that continue to facilitate the development of tissue-engineered implants for the treatment of PD and other neurological disorders.

1. Allogeneic cells can survive over many years in the brain with initial immunosuppression therapy (6–18 months) [22] but xenogeneic (porcine) implants do not [28].
2. Grafted neurons can integrate, function, and interact with the host brain in a physiological and reciprocal manner.
3. The mechanisms of graft-induced dyskinesias have been elucidated and may be eliminated.

4. Animal models have been developed to translate and scale up experimental brain implants for the clinic.
5. Surgical techniques have been developed that allow for the safe injection and implantation of cells and tissue-engineered products in the brain.
6. Trial designs and outcome measures have been developed that facilitate safety and efficacy measures in the clinic.
7. Imaging techniques have been developed to evaluate the implantation and function of the brain implants.

Cell line implants

Aside from ethical considerations, important drawbacks using primary tissues are its limited supply, its heterogeneity, and the difficulty and prohibitive costs to implement the necessary good manufacturing principles for harvesting, manipulation, storage, and later use. For example, fetal transplantation experiments for PD required fresh tissue from 4 to 8 donors resulting in procedural difficulties and poor quality control and may help explain the poor and variable efficacy outcomes in controlled trials. Therefore the ability to expand and store cells in cell banks is paramount to creating allogeneic cell alternatives to primary tissue grafts. Primary cell lines normally retain a limited number of cell cycles but allow for the proliferation of enough cells to transplant hundreds of patients from a single donor. Cultured primary cells can be expanded while retaining normal genotypes and phenotypes with normal contact inhibition and differentiation behaviors. These cells are therefore relatively safe to use, and the formation of tumors or other abnormal behaviors are relatively unlikely.

A few years ago, primary cell lines of retinal pigmented epithelial (RPE) cells derived from the retinas of organ donors were made with limited expansion capacity and were evaluated in clinical trials for PD. The RPE cells were thought to secrete L-DOPA and function by increasing the intrastriatal L-DOPA concentration and subsequent conversion to dopamine by residual dopaminergic nerve endings and glia. These cells were grown and transplanted on gelatin microcarriers to improve survival and prevent immune rejection. A report on a clinical pilot trial showed that these implants were well tolerated and safe [29]. However, a Phase II trial failed to reveal any evidence of efficacy [30] and earlier published autopsy results demonstrated poor cell survival at 6 months [31]. This approach has since been abandoned but was the first translational trial of primary cell lines grown on an artificial scaffolding for brain implantation.

In my laboratory, we were able to make the first long-term human expandable primary cell lines that contained neural progenitor/stem cell cultures capable of making various neurons or glia. These were mitogen responsive and could be isolated from various regions of aborted and adult human central nervous system (CNS) tissues and expanded for more than 1 year in vitro [32]. These cells could form the three major phenotypes of the nervous system (neurons, astrocytes, and oligodendrocytes) in vitro and in vivo and showed excellent survival without the formation of tumors in vivo. These stem cell–containing cultures were transplanted to various regions in animals and survived, integrated, migrated, differentiated, extended neurites, and arborized [33]. Even though the cells tended to retain the markers consistent with the anatomical region from which they were isolated [34], the cells could be manipulated with epigenetic and genetic factors to make specific cellular subtypes potentially useful for cell replacement implants. These studies were important in showing that neural progenitors could be expanded and manipulated, but sourcing still required tissue samples from adult or fetal brains and the cultures displayed senescence and limited life span. These types of cells were eventually implanted in clinical trials [35] of spinal cord injury, Batten's disease, and Pelizaeus–Merzbacher disease, but all three trials failed to meet the efficacy endpoints set up to support further development.

In general, all primary cell lines derived from tissue stem cells have a large but limited expansion potential and show senescence [36,37]. This may be due to the successive loss of immortal stem cells through the asymmetric division into progenitors (as seen in neurospheres) or alternatively, the stem cells themselves have a limit to their proliferation.

More recently, pluripotent stem (PS) cells consisting of either embryonic stem (ES) cells or derived from the induction of somatic cells into induced PS (iPS) cells have created sources of human cell lines that can provide an unlimited source of therapeutic cells from single donors. Especially iPS cells are currently being exploited as they do not involve the collection of cells from a human embryo and have therefore less ethical implications and could potentially also provide autologous cell lines where immune reactions can be avoided. Both cell types defy the normal senescence of primary cells and can be expanded from a single clone indefinitely without losing pluripotentiality [38,39]. From an industrial and tissue engineering perspective, this feature is extremely attractive as a single donation could give rise to a cell line source with the capacity to make all organs of the body in unlimited numbers. One major drawback of PS cell–derived products, however, is that the cell itself cannot be implanted but needs to undergo the relevant development in vitro to make suitable organ-specific cells for transplantation, for example, dopaminergic neurons for PD or islet cells for diabetes mellitus. As it is difficult to make pure cultures without retaining one or more

undifferentiated pluripotent cell, the risks of heterogeneous cell preparations and potential tumorigenesis have had to be overcome to develop pluripotent cell–derived brain implants [39,40].

The generation of functioning human dopaminergic neurons in vivo akin to those derived from primary ventral mesencephalic (VM) tissues was initially difficult to achieve. Although neurons with the dopaminergic machinery could be made from growth factor–expanded human neurospheres, genetically immortalized committed dopaminergic neuroblasts, and PS cells, it became increasingly clear that dopaminergic neurons in different areas of the brain were not the same and specific gene expressions consistent with dopaminergic neurons of the substantia nigra were necessary in order to survive and function in a similar manner to the dopaminergic neurons lost in PD. Interestingly, even though PS cells would have hypothetically needed more steps to be differentiated into dopaminergic neurons for a PD application, relatively short-step protocols that use developmental signals involved in the rostrocaudal and ventrodorsal specification of the midbrain can push PS cells into functional dopaminergic neurons as first demonstrated in a rat model of PD [41,42]. The nigral dopaminergic differentiation protocols of PS cells have now been relatively well established and clinical translation has started [17]. Studies have also identified important transcription signals involving the Lmx1a and MSX homeobox genes that when overexpressed in ES cells under the nestin promoter can yield dopaminergic neurons with markers consistent with substantia nigra neurons [43]. Transplantation of these dopaminergic neurons in a rat model of PD yields excellent survival, neurite extension, and function consistent with results from primary VM tissues. However, similar to the human ES cells [40], these cells also form tumors in vivo. Cell sorting techniques has therefore been applied and are used to purify the transplantable populations with promising results [44]. These findings may not only pave the way forward to make relevant "nigral" dopaminergic neurons from human PS cells and neural stem cell cultures in the not-too-distant future but also show the need for adult- and fetal-derived neural stem cell sources that do not form tumors.

Cell protection and regeneration implants

The use of primary tissues or cell lines in cell replacement approaches are aimed at making transplantable mimics of the cells lost in the disease process. In PD the use of chromaffin cells, dissected developing ventral mesencephalon, RPE cells, or PS cells have all been aimed at replacing or augmenting the dopaminergic function. However, primary tissues, cell lines, and genetically modified cells also produce secreted factors that can influence the nearby host cells or transplanted cells in potentially beneficial ways. Several growth factors are endogenously made by cells, including fibroblast growth factors, transforming growth factors, and interleukins that can have neuroprotective, regenerative, and antiinflammatory effects on nearby nerve cells. Custom therapeutic cell lines can also be made by genetic engineering to secrete specific growth factors such as nerve growth factor (NGF), glial cell line–derived neurotrophic factor (GDNF), and ciliary neurotrophic factor that when implanted in relevant anatomical areas can affect specific neuronal populations in neuroprotective and regenerative ways (ex vivo gene therapy). To protect the transplanted cells from immune rejection and to allow for the retrieval of the therapeutic cell implants, an encapsulated device can be used.

Cell implants secreting endogenous factors

Before expandable stem cells were available, an immortal carcinoma cell line derived from a human testicular teratocarcinoma isolated from a metastasis in a patient was used in clinical applications to treat the neurological sequelae of stroke [45]. This immortal cell line was pluripotent and could be induced to stop dividing and to differentiate into a neuronal phenotype using retinoic acid. A preparation of this cell line was investigated in the clinic for the treatment of ischemic stroke based on animal data suggesting that the postinjury transplantation of this cell line into an infarcted area could improve recovery. The mechanisms surrounding this effect were unclear but likely related to beneficial factors released from the cells. In a study in patients with lacunar stroke in a randomized controlled Phase II trials at the University of Pittsburgh, United States, the therapy with this cell line failed to meet the efficacy endpoints [46]. The transplantation of a cell line derived from a human cancer has obvious risks associated with it. Importantly, the approval of this trial demonstrated that cell transplantation for severe neurological disorders is seen as a reasonable strategy by the regulatory agency, as long as strong safety and some efficacy can be demonstrated in animal models.

Other groups are investigating the transplantation of immortal cell lines but are using genetic engineering to immortalize cells. Advances in genetic engineering have made it possible to extend the number of doublings a primary cell line can go through by inserting various oncogenes and cell cycle regulators. This allows for the selection, clonal expansion, and banking of a large number of cells. Besides the genetic modification, these cells retain otherwise normal genotypic characteristics. ReNeuron, a British biotechnology company, made immortal human neural stem cells that showed regenerative effects in stroke models [47] and a Phase I trial was

completed demonstrating safety and positive effects in stroke patients [48]. A Phase II trial is currently ongoing and results are expected in mid-2020 [11]. Similarly, StemCells Inc., used growth factor–expanded human neurosphere cell lines in a strategy to treat Batten's disease, a rare neurometabolic disorder, with the idea that the endogenous enzymes and factors made by the stem cells would have a therapeutic effect [35,48,49]. Unfortunately, this trial also failed to show efficacy. Neural stem cells derived from ES cells and fetal tissues have also been tested for spinal cord injury and are in current trials for amyotrophic lateral sclerosis, multiple sclerosis, and PD; but so far an efficacious treatment has not been demonstrated (see clinicaltrials.gov). Lastly, implanted autologous mesenchymal stem cells are being studied in clinical trials for stroke with mixed results [50] and in neurodegenerative disorders [51] with the idea that they produce endogenous growth factors and/or antiinflammatory molecules beneficial to injured tissue. The use of these cells appears safe but clinical efficacy has been hard to demonstrate.

The use of nonspecific neuroprotective and regenerative strategies based on the implantation of cells with unclear mechanisms may pose regulatory problems as the risk–benefit analyses become difficult to make. For example, even though positive results were inferred from the published trial with the human teratocarcinoma–derived cell line in stroke, the clinical data were not convincing enough to continue clinical development [46]. In this trial, no significant adverse events or tumors were reported but, if it they had occurred, a major setback for tissue-engineered brain implants could have been the result. The risk–benefit analysis is often difficult, and the predictive value and scale-up issues through animal models are not straightforward as demonstrated by several clinical trial failures despite efficacy demonstrated in animals. The regulatory agencies have therefore a real dilemma and, similar to the initial setbacks experienced in the field of gene therapy, a push to do clinical trials with poorly characterized cell preparations and mechanisms in patients desperate for a treatment may cause significant adverse events that can create setbacks for the whole field of tissue engineering. On the other hand, a too restrictive regulatory body may make the hurdle of bringing potentially beneficial but complex tissue-engineered products into the clinic too costly and difficult. These regulatory issues are hard to resolve, but as experience with cell containing implants build, it is likely that the decision-making and risk–benefit analyses will improve. Some of the clinical trials using primary autologous cells such as hematopoietic or mesenchymal stem cells derived from the bone marrow can also bypass regulatory scrutiny and only need approval by a local ethics committee. Unfortunately, this has led to the initiation of clinical trials based on very little evidence of preclinical beneficial effects causing potentially false hopes, high personal expenses, and potentially harmful side effects to patients desperate for therapy.

Cell implants secreting engineered factors (ex vivo gene therapy)

As PD involves a slow and progressive degeneration of dopaminergic neurons, a protective and/or regenerative strategy could be applied in the earlier stages of disease. Many protein factors have been shown to protect fetal dopaminergic neurons both in vitro and in vivo and one of the most powerful factors is GDNF [52]. This factor promotes the survival (neuroprotective effect) and neurite extension (regenerative effect) of dopaminergic neurons both in vitro and in vivo. Based on strong positive animal data, GDNF has been tried in humans in several clinical trials using infusion of the protein factor itself or via adeno-associated viral vectors coding for GDNF [53,54]. All trials have so far failed to meet the efficacy endpoints of the clinical cohorts, but individual patients have shown strong improvements supported by imaging and postmortem evidence that GDNF has affected the dopaminergic fibers and signaling in a positive manner. Therefore there remains strong interest to apply GDNF to patients earlier in their disease and in a manner that can increase the local striatal dose of the factor. Our approach to accomplish this goal is to use an encapsulated cell device capable of de novo synthesis of GDNF in the striatal target, which is described in more detail in the following section.

Encapsulated cell brain implants

The implantation of naked cells has the advantage of allowing for migration, integration, and the formation of neurites and synapses in replacement strategies. The migration and homing mechanisms that neural stem cells display in models of stroke and glioma tumors may also be utilized to deliver regenerative or tumoricidal agents respectively in genetically modified cells. However, naked cells cannot readily be removed and if a potent protein factor is being delivered to the brain, the inability to stop the treatment may pose a problem if untoward effects are noted or if the regenerative treatment is only needed for a limited amount of time. A device containing encapsulated cells that secrete the factor combines the advantages of cell and gene therapy with that of the safety of a retrievable device. One type of brain implant is depicted in Fig. 56.2 and consists of a recombinant cellular core attached to a polymer scaffolding and enclosed by a custom-made hollow fiber membrane that in turn is attached to a tether allowing for its placement and replacement/removal as necessary.

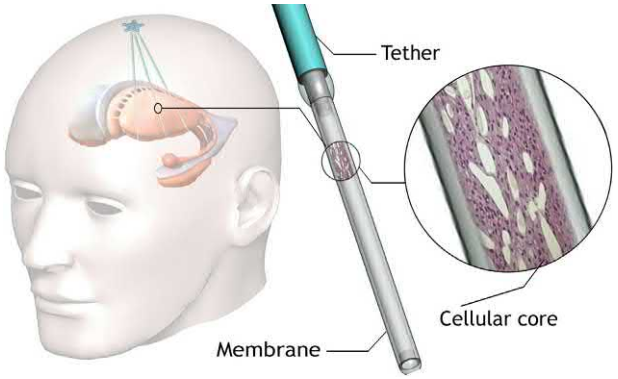

FIGURE 56.2 A schematic of the implantation for the treatment of PD is depicted and shows three separate encapsulated cell implants targeting the human putamen. The 20 mm long tips of the implants house a human immortal human cell line engineered to secrete GDNF. The cell line is grown on a three-dimensional synthetic scaffolding and this *cellular core* receives nutrients and oxygen from the surrounding brain interstitial fluid via the semipermeable *membrane* allowing for long-term function (>1 year). In turn, the factor can diffuse along the membrane in several mm wide perpendicular radii and the combination of 3–4 implants per putamen allows for therapeutic levels to reach the diseased dopaminergic nerve endings. The membrane protects the engineered cells from immune rejection. The entire device can be removed or replaced through the use of the *tether* anchored with a titanium holder beneath the skin at the skull burr hole level. The implants measure approximately 1 mm in diameter, which is similar in size to DBS electrodes currently used in the clinic. *DBS*, Deep brain stimulation; *GDNF*, glial cell line-derived neurotrophic factor; *PD*, Parkinson's disease.

These encapsulated cell implants are true tissue-engineered devices that combine genetically modified cell lines with artificial scaffolding enclosed behind an immunoprotective membrane. The polymeric membrane excludes larger molecules and cells but allows for the bidirectional passage of nutrients and transgene products. The encapsulated cells can thus be protected from immune rejection, making allogeneic or even xenogenic transplantation possible without immunosuppressive therapy. The host is also protected from the implanted genetically modified cells and the risks of gene transfer or tumor formation are greatly diminished. The tether allows for handling, implantation, and removal/replacement. These devices can be implanted intraparenchymally, intracerebroventricularly, or intrathecally depending on the application. Cellular survival and continuous production of factors have been demonstrated for at least 12 months in the brain allowing for long-term delivery of therapeutic factors [55,56]. Encapsulated devices secreting GDNF have been studied in rodent models for PD and have shown both neuroprotective and neuroregenerative effects on dopaminergic cells [57,58]. A Phase Ib trial in Alzheimer's patients was completed demonstrating safe and targeted delivery of NGF to the brain using encapsulated, genetically modified ARPE-19 cells. The cells survived for up to12 months with no evidence of surrounding brain inflammation or device displacement [56,59–61].

Other investigators have published data on using encapsulated porcine choroid plexus cells for the potential treatment of HD [62] and PD [63]. These cells are reportedly therapeutic by secreting various endogenous factors that have neuroprotective and regenerative effects. The application of the choroid plexus cells in these studies were made with an injectable microencapsulated cell configuration. In this setting the encapsulation provided immunoprotection for the porcine-derived primary choroids plexus cells. Unlike the macro-encapsulation brain implants described earlier, these injectable micro-implants would not be retrievable and may be less suited for applications in which the treatment may need to be stopped or modified. A recently completed Phase II trial for the treatment of PD with these encapsulated porcine choroid plexus cells failed to meet the efficacy endpoints [3] and it is unclear if other clinical applications are contemplated.

Controlled-release implants

Acellular synthetic polymeric brain implants that are able to deliver protein factors or other drugs to the CNS have also been developed [64–66]. These systems normally release drugs by degradation- or diffusion-based mechanisms over an extended time (weeks) but cannot sustain release over a long time (months), which is possible with cellular-based systems.

Appropriately designed, polymeric controlled-release devices have several possible applications and could for example support the survival and integration of transplanted cells. Furthermore, a polymeric system can support the sequential release of growth factors that may be necessary to fully support the stepwise differentiation of immature cells. This concept could become applicable to transplanted neural stem cells that may lack important embryonic developmental signals in the adult brain.

Combined replacement and regeneration implants

From a tissue engineering point of view, the future goal is to make replacement organs for the body that can take over the function of a failed organ or structure in an anatomically and physiologically correct manner. And even though it would be difficult if not impossible to make entirely new brains, it should become possible to not only replace cells but also to make new axonal pathways and restore the correct connections.

The transplantation of fetal dopaminergic cells to the striatum is called heterotopic transplantation. This means that the dopaminergic cells are transplanted into an anatomical region different from their normal location, which is the substantia nigra. The heterotopic implantation of dopaminergic neurons may result in the loss of important

normal innervation and feedback loops. Many transplants for PD may thus only work as simple cellular pumps that increase the striatal dopamine levels. Although simplicity is desired, an ultimate strategy to treat PD could be to transplant the dopaminergic neurons to their anatomically correct position (homotopic), regenerate the nigrostriatal axonal pathway, and induce terminal sprouting and innervation of the striatal target neurons. This would regenerate the appropriate connections and represent a more physiologic strategy.

An initial approach may be to provide survival factors to the implanted cells. Even in the most optimal VM grafts applied heterotopically in PD, the total fraction of surviving dopaminergic neurons was only about 10%–20% [67]. This required a large number of donors [4–8] to assure enough surviving cells for a clinical effect. Also with dopaminergic grafts derived from PS cells, the survival and integration may require supportive signals. The combination of dopaminergic grafts with a neuroprotective and regenerative effect of GDNF or other factors is therefore a logical idea. Experimental data indeed show that the application of GDNF delivered by encapsulated cells in combination with either rat or human VM grafts increase the survival, neurite extension, and innervation of the striatum in a rat model of PD [68,69]. Similarly, it would be expected that GDNF would have survival and regenerative effects on dopaminergic cells derived from stem cells when placed in vivo. A combined approach with dopaminergic grafts and encapsulated cell implants secreting GDNF may therefore be contemplated in future transplantation studies in PD.

A large challenge for tissue engineering approaches in the treatment of neurological disorders is the regeneration of axonal pathways. Axons between the cell bodies and their targets often extend for several centimeters in the brain and close to one meter between the brain and the lumbar spinal cord neurons in an adult. Compared to the relatively short distances that the axons had to grow during development to make the appropriate connections, the regeneration in the adult may pose a particular challenge. Fortunately, science has made progress in this area and what was though impossible only a few years ago now seems more feasible. Several molecules are now known to both promote and guide axonal outgrowth. As mentioned, GDNF is a strong promoter of axonal outgrowth of dopaminergic neurons. In addition, certain extracellular matrix proteins, such as laminin, can guide axonal outgrowth and extensive nigrostriatal reconstruction has been accomplished using bridges of striatal tissues in combination with fetal mesencephalic grafts placed in the substantia nigra [70]. The finding that the central myelin and glial scars are inhibitory to axonal outgrowth has led to the identification of various inhibitory molecules that can be manipulated in various ways with inhibitors and enzymes [71]. From a tissue engineering point of view, the combination of replacement cells with regeneration channels or scaffoldings capable of releasing survival and neurite-promoting factors and coated with molecules that facilitate axonal outgrowth may thus become a future reality. In combination with nanotechnology, synthetic bridges or living cell channels can be made that promote extensive fiber regeneration and functional restoration [72,73].

Even though there mounting data show that axonal bridges can improve axonal growth in animal models, these methods have not been applied to humans. As the cellular building blocks become better refined, it is likely that more “true” tissue-engineered brain implants will enter the clinic. These types of implants could have great potential use for regeneration in many areas of the CNS, particularly the spinal cord.

Disease targets for brain implants

As mentioned, PD has been a major target for cellular brain implants. However, many other neurological disorders should become amenable to tissue-engineered implants.

In HD, several neuronal populations slowly degenerate and cause the clinical signs of choreiform movements and progressive dementia. HD is inherited as an autosomal-dominant disease and the responsible mutation has been located to chromosome 4. Carriers of the disease can therefore be screened for and identified before the onset of symptoms. This makes a neuroprotective strategy for HD an attractive possibility, where the delivery of neurotrophic factors could prolong the symptom-free interval [74].

Cell replacement strategies have also been tried in HD [75]. Primary fetal striatal tissue transplantation for HD has been performed at a handful of centers in the world. Long-term follow up has described mild improvements in some of the implanted patients but effects have not been consistent [4,5,76]. One theoretical advantage over PD is that the transplantation for HD involves homotopic implantation, which should allow for the differentiation of the transplanted cells using normal environmental cues. However, in HD, multiple sets of neuronal populations degenerate, including both cortical and striatal neurons. The homotopic transplantation for HD may thus require more extensive regeneration of axonal pathways than in PD.

Other diseases that could be amenable to the implantation of cells within the brain are the myelin disorders. Animal experiments have shown the ability of neural tissue, purified oligodendrocytes, oligodendrocyte precursors, immortalized glial cells, and neural stem cells to remyelinate areas of demyelination [77,78].

One of the most common neurological disorders is epilepsy that affects about 1%–2% of the population.

Epilepsy is characterized by recurrent abnormal electrical discharges in the brain affecting subparts of the brain or generalizing to deeper parts in the brain resulting in unconsciousness. A subgroup of these patients has temporal lobe epilepsy that is generated by a loss of neurons and an imbalance of inhibitory and excitatory neurotransmitters in the hippocampal formation. In medically intractable cases, this disease can sometimes be treated surgically with the removal of the medial hippocampus and the abnormal area. This procedure eliminates or reduces the frequency of seizures in selected patients but involves a major surgical procedure and the ablation of normal tissue. A less invasive procedure may be to implant inhibitory cells in the seizure focus that would raise the seizure threshold [79]. This idea is supported by animal experimentation data that indicate that locus coeruleus grafts and the local delivery of inhibitory substances such as GABA can increase the seizure threshold. Preclinical studies have suggested that interneuron precursor cells derived from the medial ganglionic eminence may be a source of inhibitory GABAergic neurons perhaps providing a useful source of transplantable cells for epilepsy [80]. More recently, stem cell–derived GABAergic cells have been generated with antiepileptic effects when transplanted to animal models [81]. Furthermore, the encapsulated cell devices secreting GDNF have been shown to have strong antiepileptic effects in two different models of epilepsy warranting translation toward the clinic [82,83].

Other disease indications that may benefit from brain implant strategies include stroke, brain injury from trauma, AD, and rare disorders such as cerebellar degeneration and inherited metabolic disorders. Besides the brain, the spinal cord and retina are potential targets for similar approaches.

Surgical considerations

The surgical implantation of most brain implants involves the use of stereotactic techniques. The stereotactic method (stereotaxis) in brain surgery was established in the beginning of this century and is now well established in neurosurgical practice [84]. It involves attaching a rigid frame (stereotactic frame) to the skull followed by imaging such as MRI. Attached markers (fiducials) create a three-dimensional coordinate system in which any point in the brain can be defined and related to the frame with high precision. In the operating room the markers used during imaging are replaced with holders that guide the instruments. It is a relatively simple neurosurgical procedure often done under local anesthesia and mild sedation. The procedure is therefore safe and relatively painless. The patients are usually discharged from the hospital after an overnight observation.

Conclusion

In this chapter, various brain implants have been described that may have potential to treat PD and other neurological disorders using tissue engineering strategies. Most of the current literature describes the transplantation of various primary cells such as fetal tissue. Tissue engineering principles and cell lines have only more recently been introduced. Applications using growth factor support, genetic engineering, scaffolds, extracellular matrices, and encapsulation have all been able to improve the survival and function of the brain implant. The ultimate implants are yet to be developed and may combine stem cells, genetically modified cells, controlled delivery devices, axonal bridges, scaffolds, and encapsulated cells.

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Chapter 57

Brain–machine interfaces

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Introduction

The use of brain signals for interaction as well as for controlling robots and prosthetic devices has gained increasing attention over the last two decades. This is a rapidly emerging field of multidisciplinary research called *brain–machine interfaces* (BMIs), or *brain–computer interfaces* (BCIs), which has seen impressive achievements over the past few years—prototypes for writing messages with a virtual keyboard [1–6], playing brain games [2,7–10], and even controlling robots or wheelchairs [11–21]. A BMI monitors the user's brain activity, extracts specific features from the brain signals that reflect the intent of the subject, and translates their intentions into actions—such as closing the prosthetic hand or selecting a letter from a virtual keyboard—without using the activity of any muscle or peripheral nerve [22]. The central tenet of a BMI is the capability to distinguish between different patterns of brain activity, each being associated to a particular intention or mental task. Hence, adaptation is a key component of a BMI, because, on one side, users must learn to modulate their brainwaves so as to generate distinct brain patterns, while, on the other, machine-learning techniques ought to discover the individual brain patterns characterizing the mental tasks executed by the user. This chapter introduces the field of BMI, with a particular focus on principles for reliable and long-term operation of neuroprostheses. For a more detailed coverage of BMI, interested reader can refer to [22–24].

BMI technology offers a natural way to restore, replace, or augment human capabilities by providing a new interaction link with the outside world. In this respect, it is particularly relevant as an aid for patients with severe neuromuscular disabilities, although it also opens up new possibilities in human–machine interaction for able-bodied people [25]. Fig. 57.1 shows the general architecture of a brain-actuated device such as neuroprostheses for motor restoration and recovery. Brain activity, electroencephalogram (EEG) signals in this example, is recorded with a portable device. These raw signals are first processed in order to extract some relevant features that are then passed on to a mathematical model (e.g., statistical classifiers/regression or neural networks). This model computes, after some training process where it finds the prototypical patterns of brain activity associated to each mental command, the user's intention that is transformed into an appropriate action to control the device. Finally, visual feedback, and maybe other kinds such as haptic stimulation [26,27], peripheral stimulation [28], or intracortical microstimulation [29–31], informs the subject about the performance of the neuroprosthesis so that they can learn appropriate mental control strategies and make rapid changes to achieve the task.

This chapter is organized as follows: first, we will review the different kinds of brain signals that can be recorded as input for a BMI. Then we will discuss a series of principles to build efficient BMIs that are independent of the particular signal of choice. These principles concern the nature of electrical brain correlates more suitable to control neuroprosthetic devices and to promote motor rehabilitation, the use of machine-learning techniques, and the design of context-aware BMIs. We will conclude discussing some future research directions in the field of BMI.

Brain–machine interface signals

A BMI may monitor a variety of brain signals, such as electrical, magnetic, and metabolic. Magnetic fields can be recorded with magnetoencephalography (MEG), while

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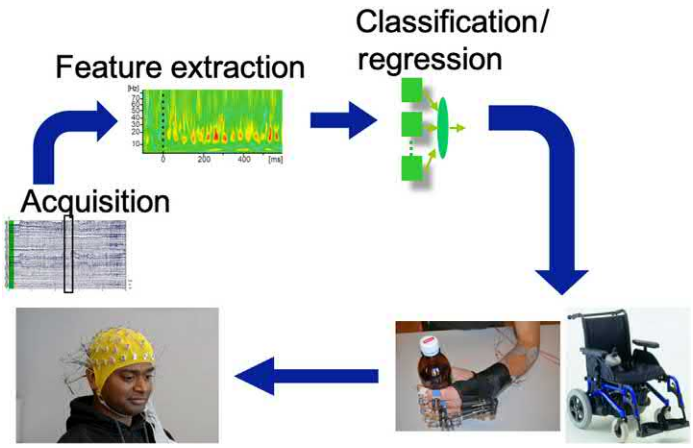

FIGURE 57.1 General architecture of a BMI for controlling devices such as a hand orthosis or a motorized wheelchair. In this case the BMI measures EEG signals recorded from electrodes placed on the subject's scalp. *BMI*, Brain–machine interface. *EEG*, electroencephalogram.

brain metabolic activity—reflected in changes in blood flow—can be observed with positron emission tomography (PET), functional magnetic resonance imaging (fMRI), and near-infrared spectroscopy. Unfortunately, MEG, PET, and fMRI require sophisticated devices that can be operated only in special facilities. Moreover, metabolic signals have long latencies and thus are less appropriate for ecological interaction. Brain electrical activity, on the other hand, can be recorded with portable devices and has an excellent time resolution—we can detect changes in brain activity at the millisecond range—that makes it the natural candidate for a BMI. We can record the electrical brain activity invasively or noninvasively. The former technique employs microelectrode arrays implanted in the brain that record the activity of single neurons (single unit activity, SUA) or of small neuronal populations (local field potentials, LFP). The overall concerted activity of neuronal populations can also be recorded (semi-) invasively with electrodes placed on the surface of the brain (electrocorticography, ECoG). Noninvasive EEG activity is recorded from electrodes placed on the scalp. It measures the synchronous activity of thousands of cortical neurons. LFP, ECoG, and EEG signals are similar in nature, although derived at different levels—microscopic, mesoscopic, and macroscopic, respectively.

Invasive approaches (LFP and SUA) carry a detailed information that may allow decoding of complex movement intentions [4,16,18,20,21,29,32–35]. However, we need to record from many electrodes and different areas to capture the global set of motor parameters, which are encoded in a rather distributed brain network. Also, implanted microelectrode arrays damage brain tissue, and so signals may not last long. Consequently, one area of active research is the design of safe biophysical interfaces that, in addition, should be ultralow power and wireless [36]. On the other hand, being recorded on the scalp, noninvasive EEG signals suffer from a reduced spatial resolution and increased noise. In particular, EEG can be contaminated with muscular—such as subtle facial movements—and ocular artifacts that, ideally, must be filtered out. Alternatively, we must ensure that the selected control features are proper brain signals and not generated partially, or completely, by non-brain sources.

Regarding the spatial resolution of EEG, it can be greatly improved by spatial filters that estimate the electrical activity originated in radial sources immediately below each recording electrode. Another way to increase the spatial resolution of raw EEG is to estimate the cortical activity from the scalp EEG, thus unraveling the contributions of different small cortical areas that are picked up and mixed by a single scalp electrode [37,38].

Analysis of human ECoG is attracting significant interest as, being semi-invasive, it blends the advantages of both approaches: high spatial resolution and simple surgery with minimal health risks. Although not largely used because of limited access to patients—who undergo neurosurgery for epilepsy or brain tumor—during short periods of time, initial results show high potential [39–43]. Long-term stability of ECoG has been demonstrated in daily use communication by humans [5].

Voluntary activity versus evoked potentials

A BMI can exploit signals associated to external sensory stimulation—such as visual flashes or auditory tones—or to endogenous voluntary decision processes—such as the onset of a movement. In the former case the brain reacts with the so-called evoked potentials. Three different evoked potentials have been mainly explored in the field of EEG-based BMI, namely, P300 [6,22,44–47], steady-state visual evoked potential (VEP) (SSVEP) [48,49], and

error-related potentials (ErrPs) [50–52]. P300 is a potential evoked by an awaited infrequent event that appears at centro-parietal locations along the midline of the scalp, independently of the sensory stimulation modality. As indicated by its name, it is a positive wave peaking at around 300 ms after task-relevant stimuli. The amplitude of the P300 depends on the frequency of stimulus occurrence—less frequent stimuli produce larger responses—and task relevance. VEPs reflect electrophysiological mechanisms underlying the processing of visual information in the brain and vary in response to changes in visual stimuli. SSVEPs are VEPs induced by a stimulus repeated at a rate higher than 6 Hz. SSVEP is composed of a series of components over the visual cortex whose frequencies are the harmonics of the stimulus frequency. Another interesting case of evoked potential is the ErrP, elicited by the neural processing of an erroneous event [53]. ErrPs are known to originate at the anterior cingulate cortex creating EEG waveforms typically composed by a first characteristic negative deflection in fronto-central regions followed by a positive peak also centrally localized. ErrPs could be used for endogenous, seamless error-correction in BCI [54–56] or even as the main BCI control modality [57,58].

Evoked potentials are, in principle, easy to pick up. The necessity of external stimulation does, however, restrict the applicability of evoked potentials to a limited range of tasks. This is particularly the case when controlling robotic devices and for motor rehabilitation. In both cases, BMI has to rely upon brain correlates of voluntary mental activity, which users can modulate at different frequency ranges—or rhythms. Populations of neurons can form complex networks with feedback loops, which give rise to oscillatory activity. In general, the frequency of such oscillations becomes slower with the increasing size of the synchronized neuronal assemblies. A particularly relevant rhythm can be recorded from the central region of the scalp overlying the sensorimotor cortex during the imagination of body movements. Correlates of imaginary movements can be recorded at any scale—microspopic, SUA and LFP; mesoscopic, ECoG; and macroscopic, EEG. Apart from their different degrees of spatial resolution, microscopic and mesoscopic signals also have a broader bandwidth (up to 300–500 Hz) than macroscopic signals (normally, less than 100 Hz).

In the case of frequencies below 2–4 Hz, we can observe slow cortical potentials (SCP). The analysis of SCP is usually done in the temporal domain. Notably, SCP measured with scalp electrodes was the basis of the first demonstration of a BMI for lock-in patients (1), who learned to modulate the SCP amplitude to control a BCI speller. The negative amplitude of SCP is related to the overall preparatory excitation level of a given cortical network—the more negative the more active. Another example of SCP that can be observed in the EEG is the so-called Bereitschaftspotential, or readiness potential—a slow negative shift over the controlateral motor cortical area starting around 400–500 ms before the onset of a movement. This makes the readiness potential particularly relevant for motor rehabilitation. However, being close to 0 Hz, its presence in single trials seems to be elusive. Nevertheless, it has been recently shown that it allows detection of self-paced reaching movement intention in single trials with humans, including stroke patients [59]. Readiness potentials are also present in intracranial LFP [60] as well as in ECoG [61]. In the former case, intracranial LFP recorded from the supplementary motor area (SMA) in an epileptic patient yielded high-recognition rates in the detection of self-paced reaching movements well before the onset. The intention to execute a self-paced movement has also been decoded from SUA in humans [62], where progressive neuronal recruitment in the SMA over 1500 ms before subjects made the decision to move could be observed. As a last observation, recent experiments have shown that scalp SCP also carries enough information to decode 3D arm trajectories [63,64].

Most spontaneous BMI rely on variations of brain rhythms in higher frequencies than SCP. In the case of EEG and ECoG, imagination of limb movements gives rise to amplitude suppression—event-related desynchronization, ERD—of Rolandic μ (8–12 Hz) and β (13–28 Hz) rhythms over the controlateral limb motor cortical area [65]. This imagination-related ERD shows different time courses in the two bands. In the μ band the ERD recovers to baseline level within a few seconds. On the other hand, the central β activity displays a short-lasting ERD followed by an amplitude increase—event-related synchronization, ERS. In particular, ERD has been largely exploited to build BMIs where subjects imagine movements of their left versus right hand or arm, or upper limbs versus feet. References are too numerous to cite them all, but see [2,3,7,9,11,12,14,15,19,26–28,40,55,66,67]. Apart from these "classical" imagined movements of hands, arms, and feet, recently researchers have also shown the feasibility of recognizing different wrist movements [68,69]. Also, as mentioned before, recent results show how it is possible to decode 3D arm trajectories from EEG [63,66] as well as basic grasping patterns such as hand closing/opening [15,70,71]. Other mental tasks such as visual attention [72,73] or even direct speech decoding [42,43,74] hold promise of a wide variety of ecological BCI designs.

Finally, because of its higher spatial resolution and broader frequency range, ECoG seems also to carry information about different motor parameters such as movement kinematics [41,61,75,76] that could provide finer

FIGURE 57.2 Example of a BMI for the restoration of grasping. The BMI is combined with functional electrical stimulation that activates the subject's peripheral nerves under the electrodes and contracts the corresponding muscles for hand closing and opening. This operation is further facilitated by the use of a light passive hand orthosis that synchronizes finger movements so as to generate natural grasping patterns. *BMI*, Brain–machine interface.

control of neuroprostheses and could greatly help in motor rehabilitation. As mentioned before, decoding of complex movement intentions has been largely demonstrated with the use of intracranial recordings (LFP and SUA) [16,18,20,21,34,35].

Although more work remains to be done in developing BCIs that decode motor intentions, their combination with functional electrical stimulation [12,15,20,21] and light exoskeletons [77,78] (see Fig. 57.2) is quite relevant for motor restoration and even rehabilitation in spinal cord injury and stroke patients (see section "Future directions").

Mutual learning

A critical issue for the development of a BMI is training—that is, how users learn to operate the BMI. Some groups have demonstrated that some subjects can learn to control their brain activity through appropriate, but lengthy, training in order to generate fixed EEG patterns that the BMI transforms into external actions [1,66,79]. In this case the subject may be trained over several months to modify the amplitude of a prespecified parameter of their brain signals, from EEG to fMRI. An alternative approach is to attempt to relieve the user from any training by using machine-learning techniques to rapidly find individual patterns of brain activity associated to the mental commands the user wants to convey [80]. The drawbacks of this approach are that the BMI usually needs to be recalibrated at the beginning of every session, or even continuously [81,82], and that many users are unable to spontaneously produce distinct brain activity decodable by pattern-recognition techniques. Most BMI systems lie in between these two extreme approaches as they need to adopt a mutual learning principle, where the user and the BMI are coupled together and adapt to each other [9].

Mutual learning facilitates and accelerates users' training process thanks to the use of statistical machine-learning techniques that both select relevant, stable features and build optimal models to decode the user's intention. Feature selection yields user-specific brain components—normally spatio-frequency features for rhythmic activity or spatiotemporal for evoked potentials—that maximize the separability between mental commands [83,84]. In addition, because of the nonstationary nature of brain signals, selected features must also be stable over time. These initial features represent those brain components that the user can naturally modulate and, via feedback received during online BMI training, learn to control quickly and voluntarily [9]. Selection of stable features can be enhanced by removing nonstationary brain sources [85].

Although there are reports of users who keep a stable level of performance over months and even years, BMIs suffer from the natural variability of brain signals due to changes in background activity and learning. This calls for the use of online adaptation techniques to keep the BMI tuned to drifts in the signals [54,81,82,86–91]. Care should be taken to coordinate the two learning agents in the BCI loop—the subject and the machine—and assure that the adapting BCI does not harm the user's learning process [9].

Context-aware brain–machine interface

Independent of the kind of BMI signal (SUA, LFP, ECoG, or EEG), paradigm (voluntary activity or evoked potential) and learning approach, users cannot sustain high levels of performance over long periods of time. This is mainly due to the natural variability of brain signals. Furthermore, EEG-based approaches yield low throughput. As a consequence, it seems that the current state-of-the-art BMI technology is insufficient for full dexterous control of complex applications such as neuroprostheses. Nevertheless, we can cope with these limitations and achieve reliable mental control by designing context-aware BMIs [92]. Such a BMI collects information about the state of the device (e.g., position and velocity of the cursor or neuroprosthesis), as well as its environment (e.g., icons in the screen or potential targets and obstacles perceived by the prosthesis' sensors), and combines it with the user's mental commands. In this approach the smart brain-controlled device interprets and executes the mental commands with respect to the context, thus enabling the performance of complex tasks even with a reduced scattered number of commands. Furthermore, any eventual decrease in mental control is compensated, up to a certain degree, by the smart device.

Examples of context-aware BMIs are neuroprostheses such as robots and wheelchairs [11,17,19,93–95], games [10], as well as smart virtual keyboards [96,97] and other assistive technology (AT) software with predictive capabilities [3]. Remarkably, in the case of control of complex devices such as a telepresence robot, this approach yields similar performance to manual control despite the fact a BMI is not a perfect control channel. This was the case not only for expert BMI users, but also for novel BMI subjects [94] and users with physical disabilities [19]. Fig. 57.3 shows our brain-controlled wheelchair.

A common form of context awareness is shared control, a technique widely used in robotics, where the user and the smart device share responsibilities in choosing the final action to be executed that best matches the user's intent. For example, it requires quite precise control to drive a wheelchair in a home environment (scattered with chairs, tables, doors, etc.). Also, it can take a long time to navigate between rooms. In a shared-control BMI the user delivers high-level mental commands such as left, right, and forward, which the wheelchair interprets based on the contextual information from its sensors to compute the actual motor commands (speed of the motors) to travel smooth trajectories and avoid obstacles. In such a way, shared control can facilitate the operation of the device by inferring goals, reducing the cognitive workload (the user does not need to care about all low-level details), inhibiting pointless mental commands (e.g., driving zigzag), preventing critical situations (e.g., collisions), and determining meaningful motion sequences (e.g., for an arm neuroprosthesis).

Future directions

Current BMI technology, in particular EEG-based, enables the operation of simple brain-actuated devices over short periods of time. Increasingly complex prototypes are being developed and demonstrated. No doubt, this will represent an important achievement for motor-disabled people. Yet, robust and natural brain interaction with more complex devices, such as neuroprostheses, over long periods of time is a major challenge.

A related issue is to develop practical BMI technologies that can be brought out of the lab and into real-world applications in order to improve the lives of countless disabled individuals. For this we need to confirm the benefit of BMI for disabled people outside laboratory conditions and, ideally, in a longitudinal manner. Only a few longitudinal studies have been conducted up to now [5,6,9,10,47,79]. In this respect, BMI technology is emerging as a promising tool for motor rehabilitation, especially after stroke [98–102]. Recent randomized clinical trials have shown the benefits of BMI-based interventions for stroke patients when combined with rehabilitation robots [98,99], virtual reality [100], and functional electrical stimulation [101]. In the latter study

FIGURE 57.3 Brain-controlled wheelchair. Users can drive it reliably and safely over long periods of time thanks to the incorporation of shared control (or context awareness) techniques. This wheelchair illustrates the future of intelligent neuroprostheses that, as our spinal cord and musculoskeletal system, works in tandem with motor commands decoded from the user's brain cortex. This relieves users from the need to deliver continuously all the necessary low-level control parameters and, so, reduces their cognitive workload.

[101], BMI patients exhibited a significant functional recovery after the intervention, which remains 6–12 months after the end of therapy and was associated with quantitative signatures of functional neuroplasticity. These results illustrate how a BMI-FES therapy can drive significant functional recovery and purposeful plasticity thanks to contingent activation of body natural efferent (motor-related brain activity) and afferent (somatosensory and proprioceptive feedback) pathways.

One of the main barriers towards wide use of BMI technology is the limitation of today's electrodes, both invasive and noninvasive. In the former case, they damage brain tissue and do not last long before they lose signal. Consequently, one area of active research is the design of safe biophysical interfaces that, in addition, should be ultralow power and wireless. In the latter case, EEG signals suffer from a reduced spatial resolution and increased noise when measurements are taken on the scalp. Significant research is taking place worldwide on algorithms to improve EEG analysis and decoding. Another of the shortcomings of today's EEG technology, which is essentially the same as 30–50 years ago, is the need for conductive gel to reduce impedance. Success of BMI, especially with recent breakthroughs, is creating a market for new sensors that will make the use of EEG-based systems much easier and robust over long periods of time. Examples of this new technology are dry electrodes that do not require any gel and can be integrated into aesthetic helmets [103], as well as skin sensors that can remain operational for months, if not years [104]. An associated challenge, however, is to develop new techniques to analyze the signals they will measure, which will probably differ from conventional electrodes.

Apart from addressing new sensors and its associated signal processing techniques, we should also develop new principles to make BMI work effectively for disabled people. In this respect, it seems natural to combine BMI with existing AT [105]. This is called a hybrid BMI—a combination of different signals including at least one BMI channel [105–107]. Thus it could not only be a combination of two BMI channels but, more importantly, also of BMI and other residual biosignals (such as electromyographic, EMG) or special AT input devices (e.g., joysticks and switches). A general architecture and a common software framework for hybrid BMIs has been already introduced [108]. Importantly, shared control (context-aware techniques) is a key component of the hybrid BMI, as it will shape the closed-loop dynamics between the user and the brain-actuated device so that tasks can be performed as easily and effectively as possible. Last but not least, novel-training paradigms and carefully designed mutual learning models could play a crucial role toward universal access to BMI technology [9,10,109,110].

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Chapter 58

Spinal cord injury

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Introduction

The spinal cord has a limited potential to regenerate after traumatic injury, due to extensive tissue destruction and a natural healing process which creates physical, cellular and molecular barriers in an attempt to preserve residual function. In this chapter, we focus on tissue-engineering approaches that use biomaterials as combinatorial therapies for spinal cord injury (SCI) repair. SCI is first described as the disruption of anatomic organization, along with its clinical and epidemiological consequences. Key tissue-engineering principles to facilitate spinal cord regeneration using biomaterial platforms in multimodal approaches are discussed. Bioengineering considerations for material fabrication in macro- and microarchitectures, and for integrated biocompatibility in animal models of SCI, are addressed. The applications of natural and synthetic polymer scaffolds with cellular and molecular functionalization, and their outcomes in regenerating spinal cord tissue after injury, are presented in detail. The chapter concludes with perspectives on the clinical translation of these technologies to improve neurologic function in patients with SCI.

Epidemiology

SCI affects more than 290,000 people currently living in the United States, with over 17,700 new cases occurring each year, primarily in men (78%), and in the prime of their lives (average age 43) [1]. While the global incidence rate is estimated to be 23 cases per million population [2], the incidence rate in the United States is more than twice than that, at 54 cases per million population [3]. A bimodal distribution of injuries by age and mechanism has been increasingly recognized. Young patients between the ages of 16 and 24 are most commonly injured in motor vehicle accidents, by acts of violence including knife and gunshot wounds, and in sporting or recreational accidents. Patients older than 65, who frequently have predisposing spinal stenosis or other structural spine disease, are injured from falls. The estimated lifetime costs attributable to the injury in individual patients varies by patient education, preinjury employment, and the extent of neurologic impairment, but range from approximately $5 million in a young, severely injured patient, to $1.2 million in an older, less affected patient [1]. The emotional, social, and psychological cost to each patient and their family is immeasurable.

Spinal cord organization

The human spinal cord is approximately 45 cm (1.5 ft) in length and is organized into 30 neuronal segments, 8 cervical, 12 thoracic, 5 lumbar, and 5 sacral segments, which span 20 bony vertebral segments from the foramen magnum to L1. The transverse diameter of the healthy human spinal cord [4] ranges between a maximum of 1.33 ± 0.22 cm ($\sim$0.5 in.) at the C5 level, to a minimum of 0.83 ± 0.21 cm ($\sim$1/3 in.) at T8. Anteroposterior measures are more uniform, ranging between 0.74 ± 0.16 cm at C5 and 0.63 ± 0.2 cm at T8. The lumbar spinal cord segments are slightly larger again than the thoracic cord, giving the length of the cord a subtle hour-glass contour. The spinal cord terminates as the conus at approximately the L1 vertebral level, immediately below the posterior rib cage, fanning out into the individual rootlets of the cauda equina.

More than 100 billion neurons are organized within this compact volume. Neurons and their supporting cells form discrete tracts and fascicle arcades to convey integrated motor commands for muscle movement from the brain to the limbs and torso (descending spinal cord tracts). Separate tracts and fascicles in the spinal cord also convey sensory information for touch, vibration, joint position, pain, and temperature from the peripheries to the brain (ascending spinal cord tracts) (Fig. 58.1). Discrete

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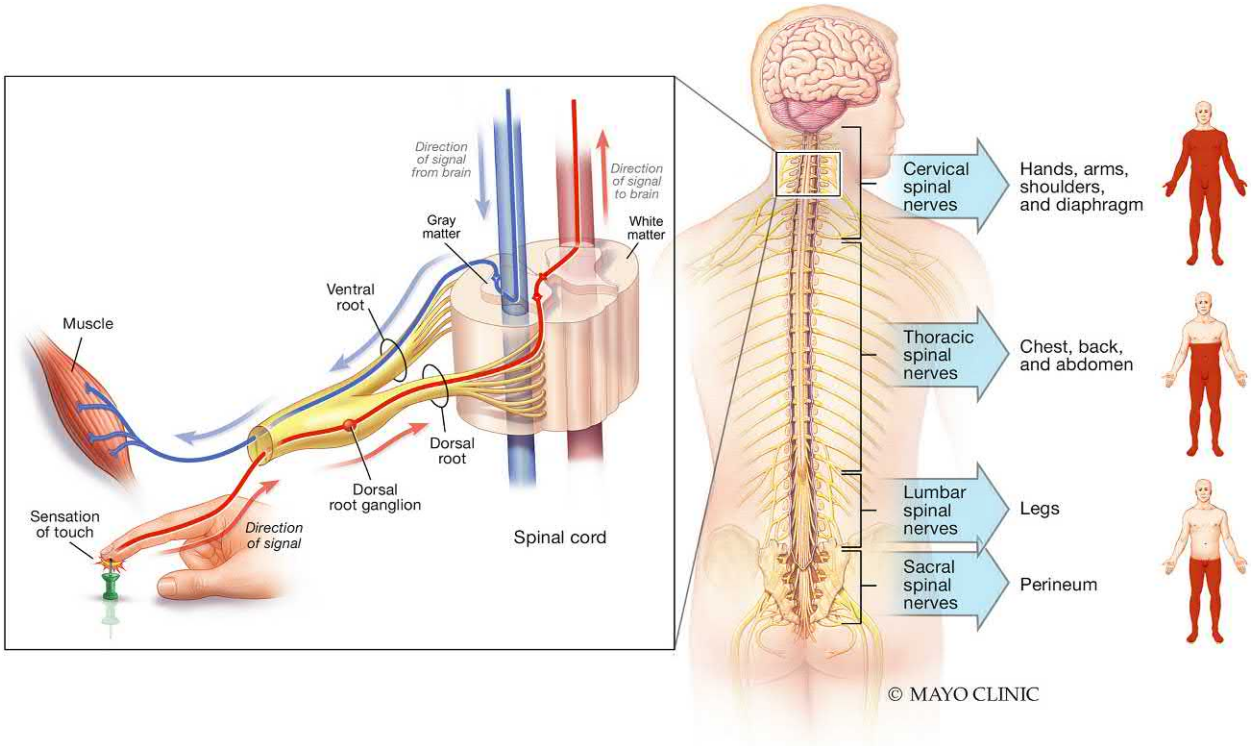

FIGURE 58.1 Organization of the spinal cord and injury sequelae. The centralized gray matter of the spinal cord contains neuronal cell bodies/nuclei, projecting interneuron axons, and glial cells. The gray matter is surrounded by white matter, containing myelinated axons which ascend and descend in sensory and motor tracts, respectively. Outgoing (efferent) messages for muscle movement exit the cord through ventral roots, while incoming messages conveying sensation (pain, temperature, vibration, limb, and joint position) enter the spinal cord via dorsal roots. The cell bodies for motor neurons are within the anterior horns of the gray matter. Bipolar neuron cell bodies for sensory neurons are outside of the spinal cord in an adjacent dorsal root ganglion, projecting a short axon into the posterior horn, which synapses to cross to the opposite side of the cord and into the ascending tract. The other axon projects distally to acquire sensory signals. The spinal cord is very densely vascularized. Clinical sequelae of an injury depend on the spinal level (cervical, thoracic, lumbar, and sacral levels), with the broad distribution of sensory and motor deficits indicated in red.

tracts are intricately interconnected within and between nearby segments by spinal interneurons. Many motor and sensory networks are organized by interneuron processing into central pattern generator systems within the spinal cord that locally integrate movements such as walking, breathing, postural control, and learned repetitive or reflexive movements [5]. These pattern generator networks can be initiated and fine-tuned by volitional control from the brain. They may also operate independently with external stimulation, including electrical spinal cord stimulation or passive limb kinetic movements, after a spinal injury that does not directly damage them [6]. Motor signals exit the spinal cord segmentally from ventral roots (efferent pathways), while sensory inputs (afferent pathways) enter the spinal cord from dorsal roots. These roots pass through the spinal bones via narrow neural foramina to converge as individual peripheral nerves.

Spinal cord injury

Any injury to the spinal cord will have dire and wide-ranging consequences in such condensed, high-priced neurologic real estate. Primary injuries typically occur due to traumatic compression of the spinal cord by vertebral column bone or disc elements that are mechanically displaced into the cord substance by violent force (Fig. 58.2) [7]. A number of injury mechanism classifications have been proposed, depending on whether there has been a spinal flexion injury (wedge fractures, vertebral body subluxation, and oblique facet dislocation), spinal extension injury (posterior column fractures), or axial load injuries (vertebral burst fractures) [8]. Less commonly, in about 25% of patients, the spinal cord is superficially lacerated or deeply transected by penetrating injuries. A minority of injuries are nontraumatic, including primary inflammatory disorders of the spinal cord (multiple sclerosis and transverse myelitis), viral infection, and neoplastic or neurodegenerative pathologies.

The spectrum of clinical phenotypes depends upon the injury mechanism, its severity, acuity, time to decompression, and the spinal level involved. Injuries affect both sensory and muscle control function and can be complete, resulting in total loss of sensation and muscle movement below the injury level, or incomplete, where limited

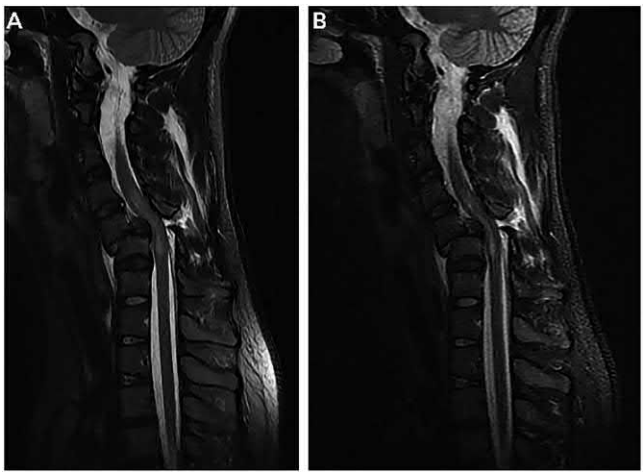

FIGURE 58.2 Traumatic cervical spinal cord injury. T2-weighted sagittal MRI of a young patient with a C5–C6 dislocated fracture and traumatic disc herniation causing severe spinal cord compression. (A) Fat-saturated imaging and (B) STIR imaging show spinal cord swelling and intermedullary signal changes consistent with acute spinal cord edema and hemorrhage. *MRI*, Magnetic resonance imaging; *STIR*, short tau inversion recovery. *Adapted from Rabinstein AA. Traumatic spinal cord injury. Continuum (Minneapolis, MN) 2018;24(2, Spinal Cord Disorders):551–66, with permission.*

function is preserved below the injury level. One-third of the patients have a complete SCI resulting in total lower limb or combined upper and lower limb paralysis, and will never recover any neurologic function. Injury in the cervical region, the most common site of injury, will produce paralysis of the arms and legs (quadriplegia or tetraplegia) (12% complete and 48% incomplete) [1]. Severe respiratory compromise occurs with higher levels of cervical injury due to damage to the innervation of diaphragm muscles, via phrenic motor nuclei located in C3–C5 spinal cord segments. These control centers may extend to the C7 level in some patients [9]. Injuries at any level, however, can affect respiratory function, through the destruction of descending motor tracts innervating thoracic, intercostal, and abdominal accessory muscles. Ascending sensory signals for coughing, vomiting, and secretion clearance reflexes are also disrupted [10,11]. Injury at the lower thoracic or lumbar level results in paralysis of the legs (paraplegia) (20% complete and 20% incomplete). SCIs at most levels will affect the autonomic nervous system, impacting upon bowel, bladder, and sexual function, as well as the vital control of blood pressure and heart rate [12]. The most common causes of death in spinal cord injured patients are related to cardiac and respiratory dysfunction, pulmonary embolus, and complications of pneumonia and septicemia [13]. Mortality rates are highest within the first 12 months after injury. Medical and surgical treatments dramatically improved life expectancy of injured patients after World War II through the 1960s. However, life expectancy has not further improved over the last 50 years despite advances in medical care and management.

Available clinical interventions

In the acute phase of injury, clinical management focuses on spine immobilization, the support of vital respiratory and cardiac/hemodynamic function, and the needs for surgical decompression and spine reconstruction. Surgical decompression within the first 24 hours of injury has been shown to improve functional outcomes in a subset of patients [12]. Pharmacologic strategies for neuroprotection, including high dose methylprednisolone [14–16], unless potentially administered within 8 hours of injury (per one post hoc analysis) [17], and the induction of epidural or systemic hypothermia [18], have not shown definitive outcome benefits in patient trials. The use of minocycline and rapamycin in clinical trials, among other experimental agents, is currently under investigation for anti-inflammatory properties and the reduction of neural excitotoxicity, respectively [19,20].

The continuum of physical, cellular, and molecular barriers to spinal cord regeneration

The spinal cord has limited potential to regenerate after damage occurs to neurons and nerve pathways. Over the first week, the acute, primary injury includes bleeding, swelling, and inflammation, through which cells of the spinal cord die as a direct consequence of local hypoxia, ischemia, and compression. Secondary acute insults may include mechanisms of necrosis via primary adenosine triphosphate (ATP) energy depletion through mitochondrial damage, calcium-induced glutamate excitotoxity, and action potential ion-pump failure. Cells may enter into programed cell death pathways including apoptosis and autophagy, or succumb to free-radical production and membrane compartment lysis via lipid peroxidation. A wound healing response is then initiated as a secondary chronic phase that aims to contain the extent of damage, rather than to recover function [20]. Regeneration requires that disrupted nerve axons regrow to traverse the site of injury and reestablish functional connections. Any neuron that suffers irreparable injury to the cell body will be permanently lost and will never be replaced. In principle, any spinal cord neuron that retains a functional cell body soma has a capacity to reextend their axons through regeneration. Viable axons remaining within the injury area (tissue-sparing) may also locally extend growth cones through sprouting, as distinguished from longer distance axonal regeneration. Functional improvement after injury then is fundamentally a balance between the

neuronal cells' ability to extend axons, sprout, and remodel synapses (neural plasticity), in the face of a hostile injury environment that is evolutionarily primed to deter regeneration.

Over the ensuing weeks to months after injury, immune-mediated tissue destruction, large cysts, extensive gliosis, and spinal cord atrophy combine to form dense physical barriers to axonal regeneration [21,22] (Fig. 58.3). Gliosis represents a process of physical scarring that is unique to the nervous system and that is coordinated by astrocytes, microglial cells, invading systemic immune cells and fibroblasts. The area of cysts and glial scarring does not contain cells or tissue that could contribute to regeneration and is consequently both a gap and a barrier to regeneration. Such areas tend to form at the injury epicenter. Adjacent to these core areas of large

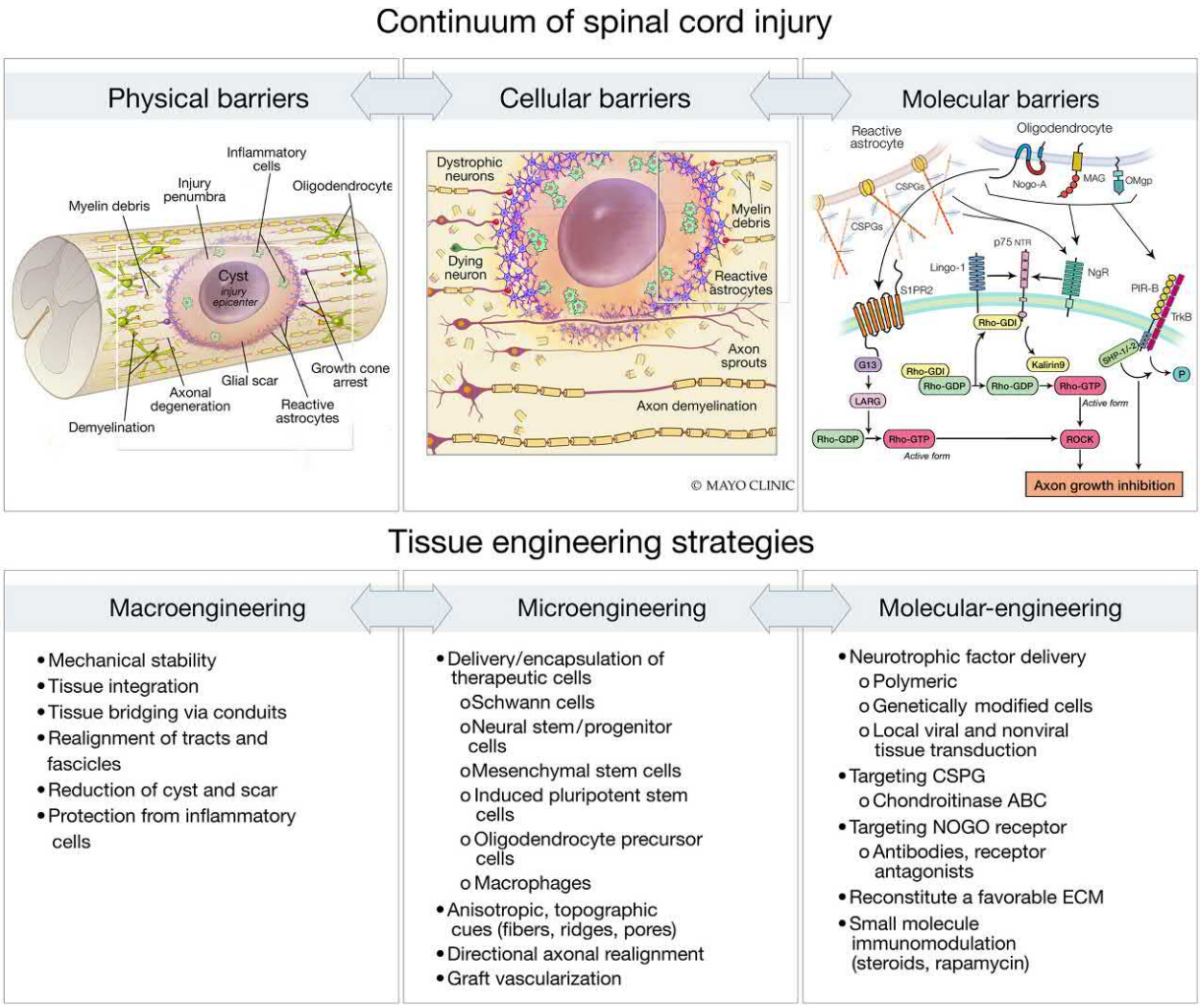

FIGURE 58.3 The continuum of spinal cord injury and corresponding tissue-engineering strategies. Physical barriers after spinal cord injury include large cysts, dense glial scarring, and spinal cord atrophy. Tissue injury occurs in a gradient of severity moving outward from a centralized epicenter of complete destruction that is walled off by the glial scar, and extending into areas of relative tissue sparing (injury penumbra). Reactive astrocytes align along the injury border to seal the area off and limit further extension through the deposition of glial scar. Damage to axons may be from the primary insult or from secondary degeneration due to oligodendrocyte injury and loss of myelination. An influx of fibroblasts and immune cells contributes to the formation of collagenous, fibrotic scarring (granulation tissue). Molecular cues that directly inhibit axonal regrowth into the injury are derived from the cellular responses. Fragments of myelin debris contain inhibitory epitopes including NOGO-A, MAG, and OMgp, which will bind to axon surface receptors and initiate protein signaling cascades that lead to growth cone collapse. Similarly, contact with CSPG molecules in the glial scar signals toward axonal growth arrest or directional changes and results in the formation of dystrophic nerve terminals. CSPGs have several molecular growth cone targets including inhibiting integrin receptor interaction with laminins, as well as binding NOGO receptors. Tissue-engineering strategies for spinal cord regeneration must address the physical, cellular and molecular barriers in parallel, through macro-, micro-, and molecular engineering. Biomaterial scaffolds are powerful platforms for such combinations of therapies and as a means for hypothesis testing and experimental observation. *CSPG*, Chondroitin sulfate proteoglycan; *MAG*, myelin-associated glycoprotein; *NOGO-A*, neurite outgrowth inhibitor A; *OMgp*, oligodendrocyte-myelin glycoprotein.

physical gaps, resident cells of spinal cord are damaged in a gradient of severity that tends to lessen with distance from the injury epicenter. Neural axons undergo degeneration, leaving behind fragments of their myelin. Damaged oligodendrocytes are unable to effectively remyelinate axons. Those axons that are at a critical threshold for survival may further be lost; those barely surviving will have poor signal conduction across the injury with incomplete remyelination and may undergo further secondary axonal degeneration [23]. Astrocytes in the injury area shift their phenotype to become hypertrophic and "reactive," adjusting in their role from neuronal health maintenance toward glial scar production and damage limitation. Fibroblasts are normally excluded from the spinal cord, except in perivascular areas. After injury, they may migrate from the peripheral nervous system and fibrotic linings of the spinal cord through disrupted tissue planes to produce additional collagen-dense scarring. Inflammatory cells, including B- and T-lymphocytes and microglial cells, coordinate to both produce tissue debris through cytotoxic responses and to clear tissue debris through phagocytosis [24].

These cellular responses themselves involve the elaboration of molecular cues that actively impair regeneration (Fig. 58.3). Myelin debris contains several protein epitopes, including oligodendrocyte-myelin glycoprotein (OMgp), myelin-associated glycoprotein (MAG), and neurite outgrowth inhibitor A (NOGO-A) [25], which will initiate signal transduction cascades within advancing axons and lead to growth cone collapse [26]. Reactive astrocytes manufacture and construct a dense network of chondroitin sulfate proteoglycans (CSPGs) [27] that become the principle components of the glial scar [28]. Contact with a CSPG network by regenerating axons leads to growth arrest, or at best, an abrupt change in the direction of axonal outgrowth. Fibroblasts produce an abundance of collagen, deposited in a dense network of fibrils that is impenetrable to axonal extension. The loss of an intact vasculature at the capillary level produces an environment that is hypoxic, acidotic, and favors the accumulation of metabolic wastes over their clearance.

These cellular responses create an environment that is devoid of natural molecular cues which axons need to survive and regrow. A number of protein factors, which are essential in the normal spinal cord as trophic and survival cues for specific neuronal populations, are lost as a consequence of cellular injury [29]. For example, nerve growth factor (NGF) is involved in the growth of nociceptive cells, brain-derived neurotrophic factor (BDNF) primarily modifies the activity of motor neurons, neurotrophin (NT) -3 supports the corticospinal tracts and dorsal sensory axons, NT-4 and -5 the proprioceptive and motor inputs, and glial cell-derived neurotrophic factor (GDNF) broadly supports the proprioceptive, dorsal sensory, ascending motor, and nociceptive neurons [30]. Each of these signals plays a critical role in the development of the spinal cord during embryogenesis; several of these factors also may also be important to mobilize new glial and neuronal cells from the stem cell niche [31]. In adulthood, insufficient concentrations of these factors after SCI profoundly influence neuronal and glial cell tolerance for the injury and their ability to survive and recover function.

During normal tissue development, cells of the central nervous system also use the extracellular matrix (ECM) as a guide for cell migration and morphogenesis [32,33]. The native ECM is a naturally occurring, three-dimensional (3D) fibrillary protein network that provides key structural support, cell adhesion sites, molecular guidance cues, and biologic signals for cell survival [30]. In injured spinal cord tissue, the native ECM is damaged or destroyed and is replaced by a provisional matrix that is devoid of laminin proteins and that is derived primarily from blood plasma to include an abundance of fibronectin [34]. Such matrices again serve primarily to limit the extension of the SCI lesion but may allow time for surviving cells, including fibroblasts and glial cells, to begin to reassemble a neuronal-specific fibrillary matrix enriched with innate ECM proteins including collagen, tenascin-C, hyaluronan, and proteoglycans [35] in perineural nets. This innate matrix may also induce the proliferation of progenitor cells in the CNS for neural tissue replacement [36] and provide binding sites for soluble factors which enhance neuronal outgrowth and survival [37].

The role of tissue engineering in spinal cord injury repair

A key principle of tissue engineering in the spinal cord may therefore be to facilitate a process of "redevelopment." It may be critical to use the introduction of biomaterials, transplanted cells, and the delivery of bioactive molecules after injury as a means to guide the spinal cord tissue back through specific developmental stages, rather than to provide more mature tissues by engraftment. The mechanisms of SCI are complex, chaotic, and occur on a continuum at gross anatomical, cellular, and molecular levels. Multiple, concurrently applied strategies aiming to address each aspect of the injury continuum are necessary to facilitate repair of the injured spinal cord (Fig. 58.3). Strategies for neuroprotection and immunomodulation may maximize the capacity for surviving tissue to contribute to repair by local plasticity and are needed in conjunction with longer distance axonal regeneration [38]. Facilitating axons to regenerate across the physical injury needs further refinement in order to impact upon neurologic function, including strategies to improve the efficiency of signal conduction through remyelination, and to

enhance the accuracy of synapse formation [39]. A new microvasculature must be regenerated in parallel [40] to support regenerating axons and glial cells and to recapitulate the primary developmental phenotype of the neurovascular bundle [41].

Viable strategies may include a functional bypass, by nerve autografts [42,43] or electronic interfaces to conduct messages to and from the brain and limbs [6]. The tissue-engineering approach is to replace the cyst/scar with tissue that promotes the development of neural tissue bridges through polymeric scaffolds to carry regenerating ascending and descending axons and facilitate functional reconnection [44]. Biomaterial scaffold technologies may be particularly useful in multimodal approaches for the investigation and treatment of SCI. Surgically implanted biomaterials can bridge the physical gaps, form approximations of viable tissue with structured order, and serve as a replacement matrix to guide axonal growth through the area of injury [45,46]. Scaffolds can also be architectural systems for the delivery of cellular and molecular therapies [47]. Moreover, scaffold technologies allow for finely tuned control of the microenvironment of the injured cord and for a powerful means to make direct experimental observations in animal models [48]. Identifying and reproducing an environment that facilitates axonal extension, that provides positive trophic and survival cues, and that reduces the influence of negative cues would significantly advance biomaterial design for SCI repair and could contribute to recovery of neurologic function in patients. Tissue-engineered replacement of discrete tracts within short segments of the cord may be feasible for those patients with complete injuries and without evidence of residual functional tissue after injury. The optimal timing of surgical placement of scaffolds in an acute, subacute, or chronic phase of injury has not been determined but should ensure that no additional disruption to function occurs as a direct consequence of the surgery.

Bioengineering for integrated spinal cord biocompatibility

Tissue engineering in the spinal cord, as in other organ systems, poses bioengineering challenges that are unique to the specific tissue environment. Biomaterials, cells, matrices, and therapeutic molecules need to have properties that are compatible within that environment in order to successfully influence spinal cord tissue regeneration. In general, bioengineering attributes that most closely recapitulate the intrinsic mechanical characteristics of the original tissue may improve regeneration. For example, the elastic modulus of the human cervical cord without pia mater was measured to be on the order of 0.04 ± 0.006 MPa, and the maximum stress to failure was recorded to be 0.062 ± 0.005 MPa in one study [49]. The authors' results also implied that the spinal cord may be softer under tensile (stretch) loading than compressive loads. In another study, the moduli of intact human spinal cord with pia intact mater and without pia mater were 1.40 ± 0.088 and 0.089 ± 0.021 MPa, respectively. Despite reinforcement from the pia mater, the modulus values for spinal cord tissue are in the range of a soft gelatin, that is, a "viscoelastic" material that has nonlinear stress−strain curves and anisotropic properties [50]. This reflects the need to develop materials and biologics that have corresponding biomechanical properties: soft materials that integrate but do not damage the adjacent tissue in the dynamic environment of a moving spinal column. Biomaterial permeability, degradation kinetics and the nature of the biproducts, and physical properties of the material including swelling, stiffness, porosity, and tensile strength are key characteristics that are specific to the polymer used and its modifications (Table 58.1). These parameters determine how the biomaterial will interact with host tissue to direct a regenerative tissue response for axonal extension and remyelination. Critical regenerative responses include provision of an ECM, engraftment of exogenous transplanted or endogenous migrating cells, vascularization, and the controlled release of molecules of therapeutic interest. To provide for biocompatibility of an engineered construct, transplanted cells ideally would be autologous, or at a minimum, derived from an inbred allogenic strain to avoid host immune rejection. The experimental design should establish the viability of implanted cells over time according to the specific delivery method employed. Protein based biologics such as neurotrophic factors should be produced from the genetic sequences of the host animal to avoid eliciting immune responses. The immunogenicity and toxicity of other nonmammalian proteins (green fluorescent protein) and chemical drugs must similarly be established.

Animal models of spinal cord injury

Animal models of complete spinal cord transection, dorsal or unilateral cord hemisection, clip or balloon compression, and computer-controlled impact contusion injuries are commonly used to develop tissue-engineering methodologies and to test experimental hypotheses for regeneration [51]. These animal models approximate the human pathology of spinal cord transection or laceration, and closed compression or contusion injuries. Each injury model requires a surgical laminectomy to access and injure the spinal cord, with the exception of balloon-catheter compression. The use of a particular injury model is largely influenced by the specific experimental aims, laboratory expertise with the technique, the intensity of animal care after injury, the animal species and size, the capacity to quantitatively assess the outcome, and the

TABLE 58.1 Tissue- engineering for spinal cord biocompatibility.

Bioengineering attribute	Influence on biocompatibility within the spinal cord
Permeability	• Is a measure of a material's capacity to transmit fluid or dissolved molecules of variable size • Cerebrospinal fluid is low in cellular nutrients in relation to blood • Permeability to a wide range of molecular sizes is crucial for engineered tissue oxygenation, nutrient supply, removal of metabolic wastes, and potential for vascularization
Swelling ratio	• Defined as the ratio of hydrated to dehydrated polymer weight • The spinal cord environment is highly aqueous • Swelling influences the maintenance of implant alignment and the potential to compress adjacent tissue or occlude a scaffold lumen space
Degradation kinetics	• The rate of material degradation influences the lifespan and structural integrity of the implant in vivo • The scaffold structure should be maintained long enough to allow for tissue in-growth in the desired conformation • Degradation may be accelerated by tissue ingrowth, deposition of ECM or metalloproteinases, and the cell type coimplanted • Slowly degrading materials are likely to become encapsulated by fibrosis through foreign body reaction • Rapidly degraded materials risk outpacing tissue ingrowth • Can be employed for controlled-release of encapsulated molecules
Degradation biproducts	• Must be nontoxic to local and systemic tissues • Should not elicit an immunogenic response • Preferably can be processed by innate catabolic pathways
Compression or elastic modulus	• Defined as the ratio of stress (force per cross-sectional area) to strain (displacement per unit length) of a material • Ideally should match or approximate that of the spinal cord • Materials should be elastic enough not to damage the adjacent spinal cord with movement
Flexural modulus	• Defined as the ratio of stress to strain in flexural deformation • Assessed by three-point bending measurements • Informs the risk of structural deformation or collapse as proportional to the length of the scaffold
Tensile strength	• Defined as the maximum stress a material can withstand before failure • Surface tensile strength influences cell adhesion, migration, and may determine cell phenotype • Will influence the ability to hold a suture on implantation if required
Porosity	• Pores of varying size will influence permeability, degradation, stiffness, tensile strength, and swelling • Influences cell adhesion and vascularization of the implant • Can improve interlocking between innate tissue and the implant • May be employed to create microreservoirs for controlled release
Surface charge	• Influences cell surface adhesion • May be employed for controlled release of oppositely charged molecules

ECM, Extracellular matrix.

need to implant a device. Each model requires rigorous training to ensure reproducibility of the extent and severity of the injury, and carries distinct advantages and disadvantages in the context of a particular application (Table 58.2).

Contusion injuries are the most commonly used, in about half of all experimental SCI publications, followed by (hemi)transection and less commonly compression injuries. The most frequently used species is rat, followed by mouse, and the primary level of injury for experimental injury is within the thoracic cord (80% of publications) [52]. While the cervical cord is the most commonly injured location in humans, experimental models of cervical injury in animals are more rarely used due to high animal mortality. Each model can provide for a wide diversity of locomotor and sensory behavioral testing, autonomic assessments, neurophysiologic recordings, and histopathologic outcome measures to determine the extent of an injury and the biologic response to the intervention. Chronic contusion or compression with spinal cord cavitation, and complete or incomplete transection injuries are the most suitable for tissue-engineering

TABLE 58.2 Animal models of spinal cord injury.

Spinal cord injury model	Advantages	Disadvantages
Impact contusion		
Weight drop Computer assisted: Infinite Horizon Impactor New York University Impactor Ohio State University Impactor Frequency of use: 44% of publications [52]	• Close physiologic correlation to common human contusion and injuries • Accurately recapitulates the progression from acute primary/secondary and chronic secondary injury phenotypes • Fine control over injury severity based upon computer settings for impactor speed and delivered energy • Reproducibility of injury within and between cohorts • Less intensive animal aftercare • Most extensively published experience	• Variability of incomplete injury • Complex injury environment for measurement of outcomes • Difficulty discriminating between spared and regenerating tissue histologically • Cannot accommodate devices • Rate of animal recovery • Cost
Transection		
Complete transection Dorsal or lateral hemisection Frequency of use: 35% of publications, in roughly equal proportion [52]	• Most amenable for biomaterial device implantation and tissue-engineering applications • Allows for the creation of confined microenvironments within scaffold compartments for measurement and observation • A reproducibly complete injury • Restoration of function is primarily modulated by regenerative responses rather than by axon sprouting from spared tissue	• Represents a rare injury type in humans • Intensive animal aftercare
Compression		
Aneurysm clip Calibrated forceps Balloon Physical spacer Frequency of use: 21% of publications [52]	• Close physiologic correlation to complex bony compression injuries in humans • Clip tension, balloon pressure, and duration may be finely controlled • Reproducibility • Percutaneous (balloon) • Injury can be calibrated over time to mimic human chronic progressive SCI as in cervical spinal stenosis	• May involve an implanted device (balloon or spacers) that introduces an additional variable

SCI, Spinal cord injury.

applications, due to practical space requirements for biomaterial implantation. Bioengineered materials may be delivered as gels to fill small cavities and tears, as gels or sponges for larger defects, or as tubular or multichanneled conduits to bridge longer gaps [53]. Feline, canine, porcine [54], and primate models [55,56] of SCI typically represent advanced preclinical studies in preparation for the translation of scaffold technologies into humans. Their advantages include similar anatomic caliber of the cord and physiologic injury responses to humans. The use of these large animal models however is often cost-prohibitive, carries ethical concerns, and requires specialized facilities and veterinary expertise. It is also important to note that all of the animal types used, other than primates, are quadrupedal. Gait coordination is significantly different between bipeds and quadrupeds.

Principles of biomaterial fabrication for spinal cord injury repair

The vast majority of biomaterials used in animal models of spinal cord regeneration are polymers [57]. One classification broadly is whether the polymer is naturally derived, or whether the polymer is chemically synthetic. Scaffold biomaterials are fabricated by dissolving the monomer units in aqueous or organic solvent. The liquid state is then polymerized through the addition of a cross-linking reagent, and chemical initiators and/or accelerators. The cross-linking reaction may be initiated chemically, thermally, or by exposure to visible or ultraviolet light, depending on the specific initiator used. The biomechanical properties of the material are primarily established during fabrication, through the choice of monomer,

the fine-tuning of monomer concentrations, the stoichiometric ratios of the initiators or accelerators, and the time allowed for polymerization. Most scaffolds are produced by some form of mold-injection, confining the monomer reaction within a preformed cast, while it is still in a pre- or early polymerized state, followed by the maturation of the polymer within the mold [46]. Scaffold channels are formed by casting the polymer over multiple linear structures, such as thin wires, polystyrene cylinders, or ice crystals. Porogens of various sizes may be added to the aqueous polymer, including salt crystals, bubbles of gas or foaming (peroxide) reactions, or with composite polymers materials. The principle for pore formation is that the polymer forms around the porogen which itself is then subsequently removed, for example by dissolving the salt crystal or composite away from the solid polymer, leaving open spaces [58]. Additive manufacturing or 3D printing is just being introduced into tissue-engineering applications for SCI, and there are few published reports in this area.

Macroengineering for spinal cord scaffolds refers to the architectural features of polymerized scaffold structures that are on the order of 100−1000 μm, matching the size of nerve tracts in the human spinal cord, and typically taking the form of longitudinal conduits. Macroengineering designs address the problem of regenerating of spinal cord tissue across physical barriers and gaps for reapproximation and realignment of the disrupted fascicular anatomy. Continuous porous structures may improve regeneration and functional recovery by mimicking the intrinsic mechanical characteristics of the original tissue [59], enabling cell-responsive attachments and allowing for greater distances to be bridged [60,61]. Porosity also allows for vascularization of the scaffold implant and may improve implant stability at the cord-scaffold interface through tissue interlocking [62] (Fig. 58.4). Open conduits provide central spaces as well as walled surfaces for host axonal extension, cell migration into the construct, and for therapeutic cell delivery. The design overall may aim to align specific spinal cord fascicles or tracts (Fig. 58.5) or to provide a multitude of channels in a less specific manner for broader coverage. Early studies established the need to optimize the size and number of channels [64] provided in the scaffold design. For example, channel sizes in poly(lactic-*co*-glycolic acid) (PLGA) scaffolds of 660 μm in diameter resulted in the formation of wider areas of fibrous scarring along the inner circumferential surface of the channel walls, and smaller areas of centralized regenerated glial and axonal tissue, than open channel sizes of 450 μm [65]. This phenomenon likely relates to differential rates of cell migration into the scaffold, and cell proliferation once within the scaffold, for fibroblast and inflammatory cells over rates of axonal extension or glial cell migration into channels. The provision of more space favors the establishment of cells that migrate and proliferate quickly, even when another cell type, such as Schwann cells (SCs), are exogenously seeded within the scaffold [41]. The scaffold channel lumen over time may then become occluded with fibrotic tissue which forms a further barrier to regeneration [66].

The ratio of open space to solid polymer must also be considered in the scaffold design. It is inefficient to provide for low open space−to-polymer ratios, such that axonal regeneration is physically blocked with excessive areas of solid polymer. If the polymer itself is rigid, the adjacent spinal cord may be damaged, and the injury extended, by wider areas of spinal cord to polymer apposition [64]. On the other hand, a high open space−to-polymer ratio has implications for the mechanical integrity of the scaffold and the risk that the conduits will collapse or buckle. Finally, increasing the number of open spaces of smaller diameters (200 μm) provides for proportionally more surface area upon which cells may attach or along which axons may extend, in the form of the inner circumferential surfaces of the channel walls [67]. This

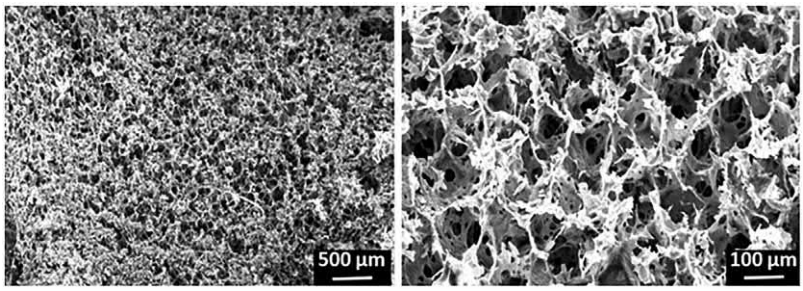

FIGURE 58.4 Scaffold porosity. A composite hydrogel fabricated from chitosan and β-glycerophosphate disodium salt as a gelling agent has a highly porous macro- and microarchitecture upon freeze drying. Porosity as a biomaterial property is critical for scaffold integration into the injury site, encapsulation of transplanted cells, and graft vascularization. *From Boido M, Ghibaudi M, Gentile P, Favaro E, Fusaro R, Tonda-Turo C. Chitosan-based hydrogel to support the paracrine activity of mesenchymal stem cells in spinal cord injury treatment. Sci Rep 2019;9(1):6402, with permission.*

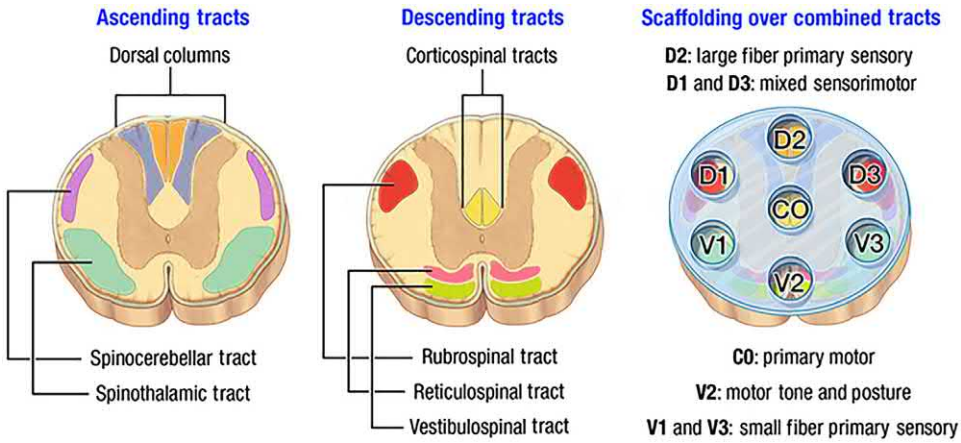

FIGURE 58.5 Macroengineering for the realignment of spinal cord tracts and fascicles. Biomaterial scaffold channels may be designed to overlap anatomically with ascending and descending tracts in the human or rat spinal cord. Individual channels therefore could support histologically and modality-specific neural regeneration. The most significant difference between the human and rat spinal cord is in the location of the descending corticospinal tracts. The rat corticospinal tracts have a centralized location in the dorsal white matter (shown here in the middle panel). A central channel (C0) (panel right) would be continuous with the descending rat corticospinal tracts and periaqueductal gray matter. A dorsal midline channel (D2) would convey axons from the dorsal columns, while dorsolateral (D1 and D3) channels overlap with ascending spinocerebellar and descending rubrospinal tracts. A ventral midline channel (V2) would associate with vestibulospinal and reticulospinal descending tracts. Bilateral ventrolateral channels (V1 and V3) are primarily ascending sensory spinothalamic tracts.

principle becomes particularly important in combinational strategies to enhance the ECM for improved cell engraftment and axonal extension.

The use of computer-assisted design, including laser etching [68] and more recent advances in additive manufacturing [69], have enabled rapid fabrication of scaffolds that precisely match the geometry of an individual spinal cord, or are biomimetic (Fig. 58.6). Matching the fit of gray–white matter interfaces, the diameter of specific neuronal tracts, and major blood vessel distribution, in addition to more general parameters such as spinal cord size overall, may help to produce scaffolds that are individualized to a specific patient's anatomy. Laser etching technologies remove areas of polymer in layers from a solid block. Inkjet or extrusion bioprinters build a scaffold structure in additive layers, which may introduce small defects consequent to the layering and droplines as the fine polymer stream is initiated. Technological advances in continuous projection printing methods have been developed for seamless scaffold production without artificial interfaces [69]. The incorporation of cells within the polymer stream for encapsulation has been achieved in applications outside of spinal cord regeneration and will likely soon be adapted for spinal cord repair [71].

Microengineering for the spinal cord refers to architectural designs of a few microns in at least one dimension [72], matching the diameters of individual or groups of cells and axons. Microengineering therefore addresses the problem of regenerating and realigning spinal cord tissue at a cellular level. Large myelinated axons in the spinal cord have diameters between 15 and 20 μm, while small myelinated and unmyelinated axons are on the order of 0.15–10 μm [73]. These design principles aim to support the anisotropic properties of the spinal cord through the linearization of the engineered tissue, recapitulating the natural anatomic tendency of 3D axons, myelinating cells and blood vessels to extend longitudinally in the planes of two primary dimensions [74]. Axonal extension is precisely guided by anisotropic polymer surfaces with etched microgrooves, embossed ridges, or aligned microfibers in addition to larger scale conduits [75]. Precision of axonal extension is necessary to optimize the likelihood that a disrupted fascicle could be rejoined together and to improve the accuracy of the intended synaptic targets. Axons and their growth cones preferentially extend along the surface interface angles that are created by ridges and grooves [68].

These topographic guidance cues have been produced on polymer surfaces by precision laser etching and by photolithography techniques (Fig. 58.7A and B). Early work demonstrated that a surface groove depth of at least 2 mm was required to linearize axonal growth over more shallow grooves or untreated surfaces [77]. Subsequently, groove widths of 30 μm, depths of 50 μm, and ridge or plateau widths of 200 μm resulted in the highest densities of bridging neurites and focal adhesion contacts over grooves of other dimensions [78]. Grooved surfaces that are coated with collagen, or laminin to provide ECM contact [79], including concentration gradients of laminin [80–82], further improve the ability to refine topographic guidance. Axons are directed to migrate from lower to higher concentrations of laminins or neurotrophic factors by molecular signaling at the growth cone through surface receptors (integrins and neurotrophic factor receptors) that adjust the axons' internal microtubule structure and

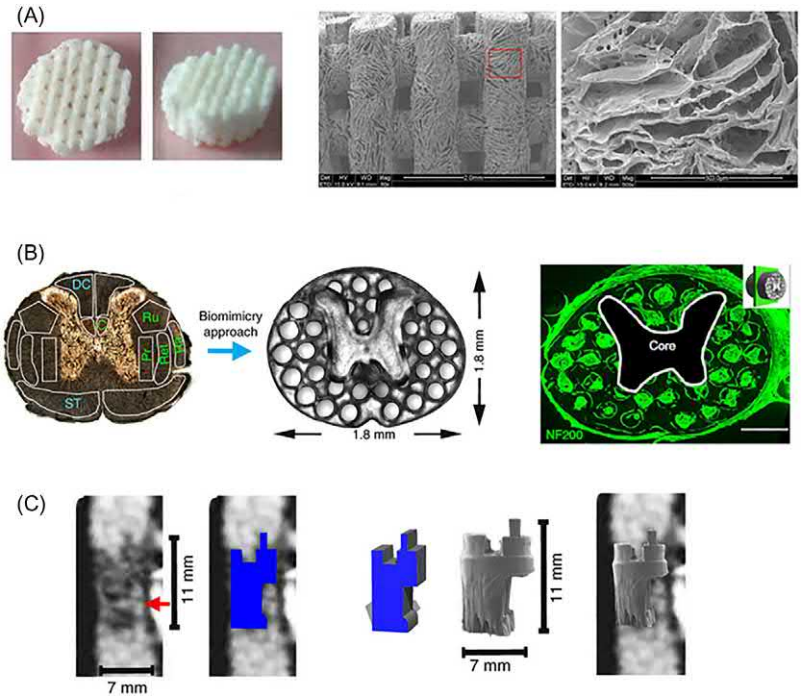

FIGURE 58.6 **Computer-assisted 3D printing.** Recent advances in additive manufacturing, building polymer scaffolds in layers with 3D printers, have enabled rapid fabrication of precision geometries for spinal cord injury repair. (A) A collagen heparin sulfate composite is printed in an open lattice design, creating larger pores between polymer columns, as well as a uniform microporous architecture within the column structure. (B) 200 μm channels are printed precisely in 3D space using a microscale continuous projection printing method and a polyethylene glycol−gelatin methacrylate copolymer, to provide conduits for specific motor (shown in green) and sensory systems (shown in blue). Since gray matter does not typically project axons following an injury, that corresponding volume was printed with solid polymer to provide for structural stability in the scaffold core. Axonal regeneration into the graft is demonstrated using neurofilament antibody labeling (NF200) after 4 weeks in vivo. (C) Computer-aided design 3D modeling of a complete mid-cervical injury cavity outlines the lesion volume, including areas of spared tissue, from a patient's T1 weighted MRI image and produces a precise scaffold shape to be printed for that individual patient. *3D*, Three-dimensional; *C*, corticospinal tract; *DC*, dorsal column sensory axons; *MRI*, magnetic resonance imaging; *Pr*, propriospinal tract; *Ra*, raphespinal tract; *Ret*, reticulospinal tract; *Ru*, rubrospinal tract; *ST*, spinothalamic tract. *Adapted from (A) Chen C, Zhao ML, Zhang RK, Lu G, Zhao CY, Fu F, et al. Collagen/heparin sulfate scaffolds fabricated by a 3D bioprinter improved mechanical properties and neurological function after spinal cord injury in rats. J Biomed Mater Res A 2017;105(5):1324−32, with permission; (B and C) Koffler J, Zhu W, Qu X, Platoshyn O, Dulin JN, Brock J, et al. Biomimetic 3D-printed scaffolds for spinal cord injury repair. Nat Med 2019;25(2):263−9, with permission.*

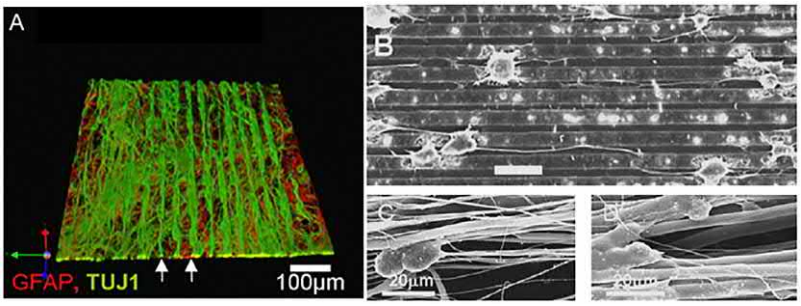

FIGURE 58.7 **Microengineering topographic cues for directional axon growth.** (A) Neurites labeled with βIII tubulin antibodies (green) preferentially extend along the inner edges of 12.5 μm grooves, on an embossed poly-ε-caprolactone (PCL) sheet with a layer of astrocytes (red, labeled with GFAP antibodies). (B) SEM imaging of PC12 neurite outgrowth on a laminin-coated PLGA with laser-etched grooves of 10 μm. (C) Neurites extend along the length of electrospun PCL nanofibers of 0.8 ± 0.7 μm (C) and 3.7 ± 0.5 μm in diameter (D). *GFAP*, Glial fibrillary acid protein; *PLGA*, poly (lactic-*co*-glycolic acid); *SEM*, scanning electron microscopy. *(A) Adapted from Sorensen A, Alekseeva T, Katechia K, Robertson M, Riehle MO, Barnett SC. Long-term neurite orientation on astrocyte monolayers aligned by microtopography. Biomaterials 2007;28(36):5498−508, with permission; (B) adapted from Yao L, Wang S, Cui W, Sherlock R, O'Connell C, Damodaran G, et al. Effect of functionalized micropatterned PLGA on guided neurite growth. Acta Biomater 2009;5(2):580−8, with permission; (C and D) from Madigan NN, McMahon S, O'Brien T, Yaszemski MJ, Windebank AJ. Current tissue engineering and novel therapeutic approaches to axonal regeneration following spinal cord injury using polymer scaffolds. Respir Physiol Neurobiol 2009;169:183−99, with permission.*

orientation along the gradient [83,84]. The addition of glial cells to patterned substrates, either to form cellular channels [85] or to be decellularized for creating a patterned native ECM [86], represent biomimetic approaches to axonal guidance.

The remyelination of axons fundamentally relies on axonal extension, as several molecular cues for SC and oligodendrocyte differentiation and maturation into fully myelinating cell phenotypes are on the axonal membrane surface. Myelinating SCs are naturally elongated and may measure between 2 and 5 μm in maximal diameter around an axon but extend between 220 and 400 μm along an axon [87]. The longitudinal extension of small blood vessels has been shown to have a directly proportional relationship to the number of adjacent axons [41], and the stereologic relationship between axon number and distance from blood vessels tends toward a Gaussian distribution.

Axons preferentially grow along the length of polymer fibers (Fig. 58.7C and D). Such fibers may be created from monomer units as the natural molecular assembly of the polymer into helices, by chemical techniques such as phase separation, freeze drying, or by electrospinning [88,89]. In vitro optimization experiments that varied poly(acrylonitrile-*co*-vinyl chloride) hollow fiber diameter from greater than 100 to 5 μm identified that filament bundles in the cellular to subcellular range (5−30 μm) improved alignment and neurite outgrowth densities [90]. The addition of laminin coating to these filaments produced longer neurite outgrowth distances. Electrospinning utilizes electrical charge to elongate threads of liquid polymer from a syringe pump source. The polymer solvent evaporates upon contact with the air, and the filament threads may be deposited in random orientations onto a flat plate or may be aligned onto rotating spindles. The diameter of the fiber is a function of the polymer concentration, the strength of the electric field, the rate of polymer draw, and the nozzle tip diameter. Aligned axonal outgrowth and glial cell alignment on electrospun fiber scaffolds have been demonstrated in animal models of SCI repair [91−93]. Polymer fibers in random orientations that are assembled into mats and sponges create a meshwork of niche environments for exogenously transplanted or migrating endogenous cells to successfully engraft into the spinal cord construct. Meshes enable a greater number of surfaces, spaces, and projections to support axonal sprouting and synaptic contacts in 3D space rather than imposing specific directionality [94].

Biomaterials for spinal cord tissue engineering: natural polymers

Biomaterials used in spinal cord tissue-engineering applications may be broadly classified into naturally derived or synthetic materials. Naturally derived polymers may be subclassified as being components of the native ECM [collagen, hyaluronic acid (HA), cell-assembled, and decellularized matrix]; components of blood (fibrin, fibrinogen); or components from marine life, insects, spiders, or other sources (agarose, alginate, chitosan, silk). Naturally derived polymers are fibrillary proteins, or complex carbohydrates such as polysaccharides or glycosaminoglycans (GAG). They form hydrogels. The use of natural hydrogels takes advantage of the intrinsic role of the polymer, as it would function as an ECM or in structural support, as well as favorable degradation characteristics. Hydrogels are particularly attractive for use in spinal cord tissue engineering, as they are typically soft materials that conform well to the injury site placement, they are porous to allow for cell engraftment and alignment [58], and they are readily permeable for aqueous exchange of cellular waste and nutrients.

Extracellular matrix polymers

Natural polymers derived from ECM proteins are intended to replace the critical role of spinal cord ECM that is lost following injury. The most commonly used ECM polymer in spinal cord scaffolds is type I collagen. Solutions of liquid monomer are easily polymerized into collagen fibrils by adjusting the solution pH or with the addition of ionic salts. Several advantages of collagen include that it is a soft material with low autologous antigenicity, and that it offers natural molecular sites for cell adhesion, migration, cell-signaling via integrin contacts, and may direct stem cell differentiation [95,96]. The collagen material by itself, however, was shown early on to have a limited capacity to support axonal extension [97,98]. Indeed collagen accumulation in scaffold channels, as deposited by the scaffold material, by migrating fibroblasts and as a foreign body response to scaffold placement over time (particularly type IV collagen deposition), is a primary barrier to axonal regeneration [66,99]. As a naturally fibrillar protein, collagen fibers scaffolds were developed to have parallel longitudinal orientations along which axons extend (Fig. 58.8). Collagen scaffolds may be more effective therefore as functionalized matrices to deliver cells or to elute molecules of therapeutic interest in single polymer or copolymer, composite systems [71,101]. For example, the covalent modification of collagen with the addition of laminin gradients provided for improved, directional axonal extension along collagen fibers in vivo [102]. Collagen is also a thermoresponsive matrix, in that it may be delivered to the spinal cord as an injectable solution which then gels or polymerizes at physiologic temperatures within the injury site [102]. Controlled release of neurotrophic factors that are in solution with the collagen

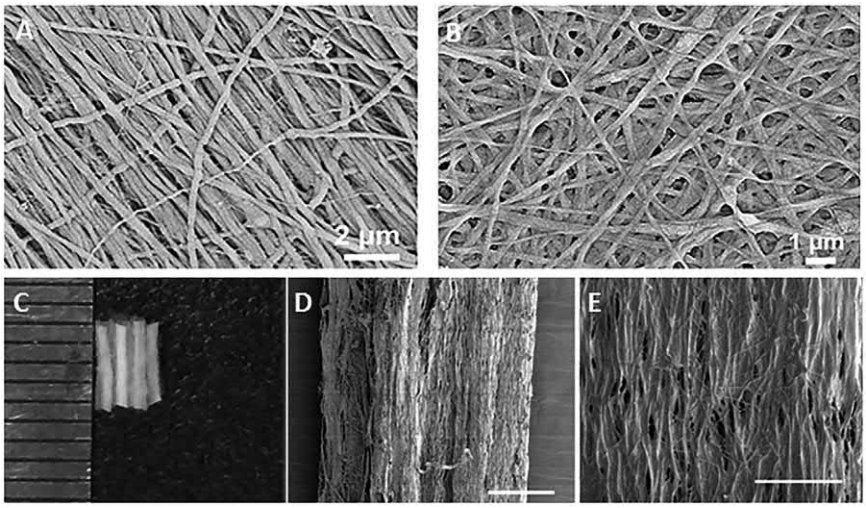

FIGURE 58.8 Collagen scaffolds. As a natural fibular protein, collagen has been a versatile material for spinal cord tissue engineering. (A) Collagen may electrospun into aligned or (B) randomly oriented fibrous mats which are then formed into tubes for surgical placement. (C–E) Linear ordered collagen scaffolds which incorporate collagen binding domains as linkers have been used extensively for the controlled release of neurotrophic factors, neutralizing antibodies and small drug molecules. *(A and B) Reproduced with permission from Liu T, Houle JD, Xu J, Chan BP, Chew SY. Nanofibrous collagen nerve conduits for spinal cord repair. Tissue Eng, A 2012;18(9–10):1057–66; © Mary Ann Liebert, Inc. New Rochelle, NY. (C–E) from Fan C, Li X, Xiao Z, Zhao Y, Liang H, Wang B, et al. A modified collagen scaffold facilitates endogenous neurogenesis for acute spinal cord injury repair. Acta Biomater 2017;51:304–16, with permission.*

has improved axonal counts and locomotor function after injury, with the rates of elution dependent upon the degradation of the collagen [103]. Collagen-binding domains have been extensively employed to covalently tether a number of small molecules and growth factors for sustained elution from linearly ordered collagen scaffolds [104,105] (Fig. 58.7) (Table 58.3).

HA is a primary GAG component of the native ECM of the spinal cord and brain [227] and, as such, was initially considered to be a good candidate to support axonal regeneration after SCI. Performance of HA as a physical substrate for axonal extension in vivo has been modest [228]. The principle uses of HA have evolved as injectable copolymer systems for drug delivery and for the encapsulation of cells [133]. The advantages of using HA in both applications include its low immunogenicity and the ability to fluidly conform to the injury defect and fill the lesion cavity with the therapeutic intervention. In addition, HA may modify astrocyte reactivity around the injury site, reduce the local extent of CSPG deposition at the injury–polymer interface [229], and limit the evolution of secondary injury mechanisms through neuroprotective effects [230] including reduced microglial cell activation [231] and subarachnoid inflammation [232].

Acetate-modified HA and methylcellulose (HAMC) copolymers have been developed with the distinctive quality of gelling at ambient and physiologic temperatures and then liquefying when subjected to sheer forces, such as within a needle and syringe, for in situ delivery [134,233]. Early applications included the sustained release of collagen embedded epidermal growth factor [135] and erythropoietin [136], enhancing neuroprotection, reducing cavity size, increasing neuron numbers, and improving tissue penetration of the therapeutic by prolonging the bioavailability following spinal cord clip compression (Fig. 58.9). The intrathecal HAMC system has been shown to have remarkable versatility for fine tuning of release and elimination kinetics of therapeutic molecules [138], through small molecule encapsulation (anti-NOGO-A) [139], PEGylation of fibroblast growth factor-2 (FGF-2) [137], NT-3, and small molecule delivery from slowing-eluting nanoparticles [140–142]. The system has subsequently been adapted and characterized for the delivery of therapeutic cells to the injured spinal cord [234], including neural stem cells (NSCs) [143], induced pluripotent stem cell (iPSC)–derived oligodendrocytes [144] (Fig. 58.9), and embryonic stem cell–derived astrocyte ECM [145].

A variety of other HA-derivative composites have been shown to support SCI repair. Strategies for the replacement of growth factors, ECM, and remyelinating cells have included using sodium hyaluronate microparticles encapsulating ciliary neurotrophic factor [235], hyaluronan tetrasaccharide [236], HA incorporating poly-L-lysine and mesenchymal stem cells (MSCs) [237,238], hydroxyphenyl HA modified with RGD peptide and fibrinogen [146], and thiol-modified HA for oligodendrocyte precursor cell (OPC) delivery [147]. Composite chitosan and HA nanoparticles have been shown to be capable of nonviral poly(ethylene imine)

TABLE 58.3 Applications of natural polymer scaffolds in spinal cord injury repair. Additional references for collagen [70,94,100,106–132].

Natural polymers	Polymer structure	Architecture	Functionalization	Animal model	Examples of applications for spinal cord repair
Natural hydrogels					
ECM					
Collagen, type I	Glycine-*X*–*Y* polypeptide fibrils	Gel		CT	Collagen gels as extracellular support growth matrix [97,98]
		Gel	NT-3, BDNF	HS	Collagen gels with embedded neurotrophic factors [103]
		Sponge	ECM peptide, MSCs	CT	Honeycomb collagen sponges for axonal alignment and extension [102,106], and nanofibrous hydrogel composites [107]
		Filaments		CT	Collagen filament bridges as growth substrates [91,92,108]
		Spheres	ESC-NPC	HS	Sphere encapsulation of differentiated neural progenitor cells [95]
		Filaments	NTF, SM, NSC, MSC	HS, CT	Linear ordered collagen scaffolds with collagen-binding HGF, BDNF, NT-3, EGF receptor antibody with NSCs [100,104,109–111]
		Filaments	NTF, SM, NSC, MSC	HS, CT	Linear ordered collagen scaffolds with collagen-binding BDNF, NT-3, cAMP, ephrinB3, Sema4D, and NOGO-neutralizing proteins, EGFP antibody (Cetuximab) and Taxols [112–117]
		Conduit	SC		SC delivery [118]
		Conduit	OES	HS	Longitudinally oriented, microstructured scaffold in cervical hemisection model [119,120], OES delivery [121]
		Fibers			Electrospun nanofibers and stem cell differentiation [94] + PCL (synthetic)
		Fibers	ChABC, NTF		Electrospun nanofibers for delivery of chABC and NT-3 [122]
		Conduit	Laminin, NSC, NTF	CT	Cross-linked, longitudinally porous scaffold with laminin, NSCs, and GDNF elution [123]
		Conduit	sNGR, ChABC, MSC	HS	Cross-linked, longitudinally porous scaffold with NOGO receptor protein, ChABC [124]
		Nanofibers		HS	Spiral conduit of comprised of nanofibers [93]
		Conduit	NTF, SC	CT	Multichannel scaffold eluting PEGylated transfection vectors for NT-3 elution [125]

(*Continued*)

TABLE 58.3 (Continued)

Natural polymers	Polymer structure	Architecture	Functionalization	Animal model	Examples of applications for spinal cord repair
		Conduit		CT	Composite scaffold with micropatterned porosity [126]
		Conduit	MSC	HS	Adipose-derived MSC delivery [127]
		Filaments		CT	Collagen filament bridge [128]
		Gel	NTF	C	Gels for intrathecal drug delivery (EGF and FGF-2) [129]
		Gel	NTF	CT	Growth factor infused matrix for synthetic polymer channels (FGF-1, NT-3) [130]
		Conduit	VEGF	CT	Collagen-binding VEGF [105]
		Gel	DNA	HS	Collagen coating of PLGA for DNA lipoplex delivery [131,132]
		Sheet	ECM		Laser micropatterned guidance gradients with incorporated collagen or laminin peptides [68]
		3D printed conduit	Composite	CT	Collagen–chitosan [71] and collagen heparin sulfate composites [70]
Hyaluronic acid	Disaccharide units of glucuronic acid and *N*-acetylglucosamine	Gel	NPC, OPCs	C	Collagen hydrogel delivery of OPCs and NPCs [133]
		Gel	NTF	CC	Methylcellulose (HAMC) copolymer injectable system [134] for EGF [135], erythropoietin [136], conjugated FGF-2 [137]
		Gel	NT-3, anti-NOGO-A		Systems of controlled release from HAMC [138,139], including nanoparticles [140–142]
		Gel	NTF, NCS, iPSC, ECM	CC	HAMC for NSC and PDGF-A delivery [143] or iPSC-derived oligodendrocytes [144]
		Gel	ECM	CC	HA for ESC-derived astrocyte ECM delivery [145]
		Gel	ECM	HS	HA with ECM peptide and fibrinogen composites [146]
		Gel	OPC	EB demyelination	OPC transplantation in thiol-functionalized HA [147]
Decelluarized ECM		Gel	ECM	HS	Acellular solubilized porcine spinal cord ECM [148]
		Gel	ECM	C	Acellular, solubilized peripheral nerve ECM for SC transplantation [149]
		Surface	ECM		Topographic surfaces of micropatterned fibroblast [86] or ESC-derived astrocyte ECM [150]

(*Continued*)

TABLE 58.3 (Continued)

Natural polymers	Polymer structure	Architecture	Functionalization	Animal model	Examples of applications for spinal cord repair
Marine and insect life					
Agarose	Linear polysaccharide of	Gel			Agarose gels as growth matrices [151–153]
	b-D-Mannuronic acid and *a*-L-guluronic acid	Gel microspheres	CNTF		Microencapsulation of neurotrophic factors [154]
		Gel	NGF		Agarose gels with neurotrophic gradients [83]
		Gel	BDNF	HS	Thermoresponsive, conformal gel with neurotrophin integration [155]
		Gel	ECM		Guidance gradients with incorporated laminin [81]
		Gel with nanoparticles	SM	C	Localized steroid delivery with agarose embedded PLGA nanoparticles [156]
		Gel	ChABC	HS	Injectable agarose microtubules releasing thermostabilized ChABC [157]
		Conduit	NGF, ECM		Fabrication of linear guidance pore scaffolds (freeze-dried agarose) with ECM, eluting NGF [158]
		Conduit	BDNF	Cervical HS	Freeze-dried, uniaxial multichannel scaffolds secreting BDNF [159]
		Conduit	MSC, NTF, Lenti-NTF	Cervical HS	NTF-eluting templated agarose with MSCs, preconditioning lesion, lentiviral NTF delivery to the adjacent spinal cord for long-tract axonal regeneration [160]
		Conduit	MSC, BDNF	CT	Templated agarose with BDNF-secreting MSCs for motor tract regeneration [67,161]
		Conduit	BDNF		Templated agarose with hydrogen-bonded BDNF [162]
		Conduit	NPC	CT	Templated agarose with neural progenitor cells (compared to 3D printed polyethylene glycol–gelatin methacrylate scaffolds) [69]
Alginate	Linear polysaccharide of	Gel	ECM		Gel with laminin or YIGSR peptide surface ligand modification [163]
	b-D-Mannuronic acid and *a*-L-guluronic acid.	Gel	GDNF	HS	Injectable alginate hydrogels with GDNF microspheres [164] and VEGF nanoparticles [165]

(*Continued*)

TABLE 58.3 (Continued)

Natural polymers	Polymer structure	Architecture	Functionalization	Animal model	Examples of applications for spinal cord repair
		Gel	EGF, FGF	BC	Injectable alginate hydrogels for EGF and FGF delivery [166]; delivery of RhoA inhibitor [167]
		Gel	ECM, OEC, SC, MSC		In vitro cell encapsulation into alginate hydrogel [168]
		Gel	MSC, macrophages	C	Microencapsulation of MSCs [169] and macrophages [170]
		Gel	BDNF	Cervical HS	Cell encapsulation of BDNF-secreting fibroblast cells [171]
		Gel	NTF	C	Gel encapsulation of Wnt3a-secreting fibroblasts [172]
		Sponge		CT	Freeze-dried alginate sponges as neurite growth substrate [173]
		Conduit	ECM, NT-3, ChABC		Freeze-casted chitosan-alginate composites [174], releasing NT-3 and chondroitinase ABC [175]
		Conduit	MSC, BDNF	Cervical HS	Capillary hydrogels with linearized anisotropic design, MSCs and BDNF delivery [176,177]
		Conduit	ECM, Astrocytes	Cervical HS	Capillary hydrogels with linearized anisotropic design for ECM and astrocyte delivery [178]
		Conduit	SC, BDNF	Cervical HS	Capillary hydrogels with linearized anisotropic design for SC and adeno-associated virus expressing BDNF [179]
Chitosan	Copolymer of *N*-acetylglucosamine and *N*-glucosamine	Conduit			Chitosan scaffolds supporting cell adhesion and growth [180]
		Sheets, fibers	SC		Schwann cell encapsulation [181]
		Conduit		CT	Chitosan conduits in long term regeneration [182]
		Gel		Cervical HS	Thermogelling chitosan-lactate hydrogel [183]
		Gel		HS	Fragmented chitosan suspension as a hydrogel [184]
		Gel		CT	Topical chitosan for neuroprotection [185]
		Gel	NSC, INF-gamma		NSC encapsulation and differentiation [186]
		Gel	MSC	CT	Chitosan hydrogel MSC encapsulation [63]
		Conduit	NPC	CT	NSC differentiation [187]
		Conduit	NPC	CT	Extra and intramedullary tissue bridge conduits with NSC differentiation [187–189]

(*Continued*)

TABLE 58.3 (Continued)

Natural polymers	Polymer structure	Architecture	Functionalization	Animal model	Examples of applications for spinal cord repair
		Conduit	NPC	CT	Protein elution from embedded PLGA microspheres [190]
		Conduit	NT-3, NPC	CT	Elution of NT-3 for endogenous NPC differentiation [191–193], and robust validation study [194]
		Conduit	NPC	CC	NPC delivery and differentiation [195]
		Conduit	NPC, cAMP	CT	NPC differentiation with cAMP eluting PLGA microspheres [196]
		Conduit	MSC	C	MSC encapsulation [197]
		Sponge	OPC		Injectable sponge encapsulating OPCs for remyelination [198]
		Conduit	NT-3	HS	NT-3 release in large animal primate model [199]
		Nanoparticles	siRNA, miRNA	Cervical SubQ injection	microRNA and siRNA delivery for microglial immunomodulation [200,201]
Silk	Fibroin polypeptide	Conduit	ECM	HS	Silk-laminin composite conduits [202] 27474892
		Conduit	GDNF, NGF, MSC	HS	Silk conduit with alginate microspheres releasing NTF cells [203,204]
		Conduit	NPC, NT-3	CT	NT-3 releasing silk film with a PCL conduit delivering NPCs [205]
		Conduit	NPC	HS	Recombinant silk spidroin microfibers as copolymer conduits with NPCs [206]
Blood-derived					
Fibronectin	Plasma glycoprotein dimer	Mat	TGF-β antibody	HS	Fibronectin mats for immunomodulation using anti-TGF-β antibodies [207]
		Mat	BDNF, NT-3	HS	Fibronectin mats for oriented axonal growth and neurotrophic factor release [208–210]
		Mat	Fibrin peptide	HS	Mat implantation with additional soluble fibronectin peptide (PRARIY) for neuroprotection [211]
		Gel		HS	Injectable fibronectin-fibrin composite [212]

(*Continued*)

TABLE 58.3 (Continued)

Natural polymers	Polymer structure	Architecture	Functionalization	Animal model	Examples of applications for spinal cord repair
Fibrin	Fibrillar glycoprotein polymer	Scaffold		HS	Fibrin scaffolds for axonal regeneration and inhibition of gliosis [213]
		Scaffold	NT-3, GDNF, NGF	CT	Neurotrophic elution from heparin complexes, NT-3 [214,215], GDNF [216] and NGF [217,218]
		Scaffold	ESC	HS	Murine embryonic stem cell–derived neural progenitor cell differentiation [219,220] and transplantation [221]
		Scaffold	ESC, NT-3, PDGF	HS	Combined NTF controlled-release and ES differentiation [222,223]
		Scaffold	ESC, NT-3, PDGF,	HS	Progenitor motor neuron enrichment and transplantation [224,225]
		Scaffold	ChABC, NEP1-40	HS	ChABC and NOGO receptor antagonism with progenitor motor neuron transplantation [226]

BC, Balloon compression; *BDNF*, brain-derived neurotrophic factor; *C*, contusion; *cAMP, cyclic adenosine monophosphate; CC*, clip compression; *ChABC*, chondroitinase ABC; *CNTF*, ciliary neurotrophic factor; *CT*, complete transection; *EB*, ethidium bromide; *ECM*, extracellular matrix; *EGF*, epidermal growth factor; *ESC*, embryonic stem cell; *FGF*, fibroblast growth factor; *GDNF*, glial cell-derived neurotrophic factor; *HAMC*, hyaluronic acid and methylcellulose; *HGF*, hepatocyte growth factor; *HS*, hemisection; *INF*, interferon; *iPSC*, induced pluripotent stem cell; *MSC*, mesenchymal stromal cell; *NGF*, nerve growth factor; *NOGO*, neurite outgrowth inhibitor A; *NPC*, neural progenitor cell; *NSC*, neural stem cell; *NT-3*, neurotrophin-3; *NTF*, neurotrophic factor; *OES; olfactory ensheathing cell; OPC*, oligodendrocyte precursor cells; *PDGF*, platelet derived growth factor; *SC*, Schwann cell; *siRNA*, small inhibitory RNA; *SM*, small molecule; *sNGR*, soluble NOGO receptor; *TGF*, transforming growth factor; *VEGF*, vascular endothelial growth factor.

DNA transfection [239], lentiviral transduction [240], and small-inhibitory RNA delivery [241] (Table 58.3).

Cell-assembled ECM is produce by whole tissues or by confluent cell cultures that are subsequently subjected to a decellularization procedure with gentle detergents. While the cells themselves are removed, the native ECM remains intact on the polymer surface, with advantages of being a complex 3D matrices with multiple protein and trophic components that may better recapitulate the original tissue [242]. Whether decellularized ECM is truly inert with respect to residual antigen has appropriately been the subject of debate [243]. Early applications of decellularized ECM for spinal cord tissue engineering have focused on using injectable hydrogel systems with ECM solubilized from porcine spinal cord, brain or optic nerve [244,245]. Porcine spinal cord ECM has been shown to be a favorable substrate for axonal ingrowth into a spinal cord hemisection lesion in vivo [148] but had disadvantages of rapid degradation rates. Acellular, solubilized ECM derived from peripheral nerve tissues (Fig. 58.10) supported transplanted cell survival and axonal growth when engrafted with SCs following a thoracic contusion injury [149]. Recent in vitro studies have shown that substrates with ECM from decelluarized protoplasmic astrocytes, in turn derived from mouse embryonic stem cells [150], as well as micropatterned fibroblast-derived ECM [86], are effective topographic surfaces for neurite outgrowth (Table 58.3).

Polymers from marine or insect life

Agarose is a naturally linear polysaccharide derived from seaweed, which is cross-linked through melting or temperature gradient change through hydrogen bonding. Agarose scaffolds have been used extensively in spinal cord repair strategies. They have the advantages of low antigenicity, a similar gelatin-like consistency to the spinal cord, and slow biodegradation rates. The ability for agarose as a material to innately support axonal extension along its surface is limited [151–153]. Agarose is also

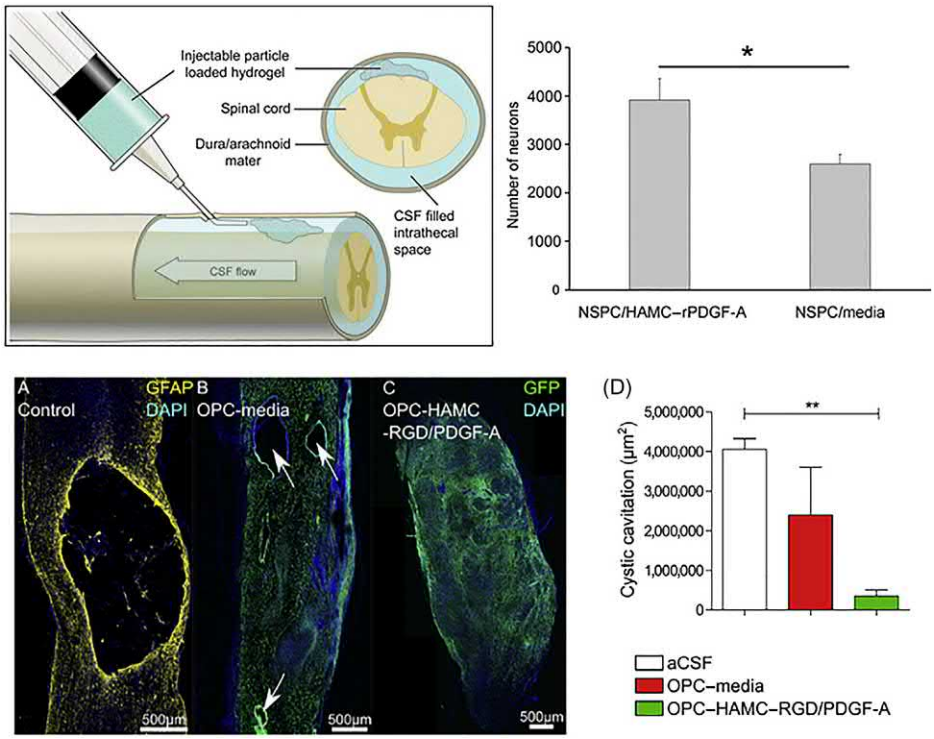

FIGURE 58.9 Hyaluronic acid. Composite polymers of HAMC form a unique injectable delivery system for neurotrophic molecules, extracellular matrices, and therapeutic cells. The hydrogel may be delivered onto the surface of the spinal cord (upper panel left), into the substance of the cord adjacent to an injury, or may be used to fill an injury cavity. Hydrogel delivery of NSPC along with chemically conjugated biotin-recombinant platelet derived growth factor-A after clip compression injury promoted neuronal sparing and improved absolute numbers of neurons surrounding the lesion site relative to NSPC alone (upper panel right). (Lower panel, A–D) iPSC-derived OPCs, with PDGF-A and an RDG motif peptide, reduced cystic cavity size after clip compression and attenuated teratoma formation when transplanted in HAMC. *HAMC*, Hyaluronic acid and methylcellulose; *iPSC*, induced pluripotent stem cell; *NSPC*, neural stem/progenitor cells; *OPCs*, oligodendrocyte precursor cells; *PDGF-A*, neurite outgrowth inhibitor A. *(Upper panel left) Adapted from Baumann MD, Kang CE, Stanwick JC, Wang Y, Kim H, Lapitsky Y, et al. An injectable drug delivery platform for sustained combination therapy. J Control Release 2009;138(3):205–13, with permission; (upper panel right); adapted from Mothe AJ, Tam RY, Zahir T, Tator CH, Shoichet MS. Repair of the injured spinal cord by transplantation of neural stem cells in a hyaluronan-based hydrogel. Biomaterials 2013;34(15):3775–83, with permission; (lower panel, A–D) from Fuhrmann T, Tam RY, Ballarin B, Coles B, Elliott Donaghue I, van der Kooy D, et al. Injectable hydrogel promotes early survival of induced pluripotent stem cell-derived oligodendrocytes and attenuates longterm teratoma formation in a spinal cord injury model. Biomaterials 2016;83:23–36, with permission.*

thermoresponsive but typically gels at temperatures lower than physiologic 37°C. This property has been useful in designing injectable in situ constructs that conform well to the spinal cord defect upon rapid cooling [155]. Gels have been used for delivery of neurotrophic factors [83,154], for localized methylprednisolone delivery from gels embedded with drug-eluting nanoparticles [156], and thermostabilized chondroitinase ABC (chABC) enzyme from an agarose hydrogel microtubule delivery system [157] for CSPG degradation in vivo.

In a series of publications that have been fundamental to the field, agarose biomaterials have been developed that provide longitudinally oriented scaffold templates for axonal extension, with parallel channels ranging from 120 to 200 μm in diameter. The scaffolds are templated by either a freeze–drying method which creates longitudinally oriented ice crystals as porogens [158,159], or by casting the agarose over polystyrene microfibers [67]. Templated agarose scaffolds have been functionalized with ECM and neurotrophic factors [158,159], including integrated gradients, and modified for small molecule elution. These studies have been more recently extended to combinatorial treatments, including preconditioning sciatic nerve compression lesions and lentiviral delivery of NT-3 into the spinal cord adjacent to the scaffold to achieve long-tract axonal regeneration through the injury site [160]. Templated agarose has also been used to deliver MSCs genetically modified to secrete BDNF [161], and hydrogel-bonded BDNF [162] (Fig. 58.11). Recently, neural progenitor cell delivery in templated agarose was directly comparable to delivery in a synthetic polymer scaffolds fabricated by 3D printing [69] (Table 58.3).

Alginate is a linear polysaccharide derived from brown seaweed [246], which is cross-linked with sodium or calcium ionic salts. It has many of the same advantages

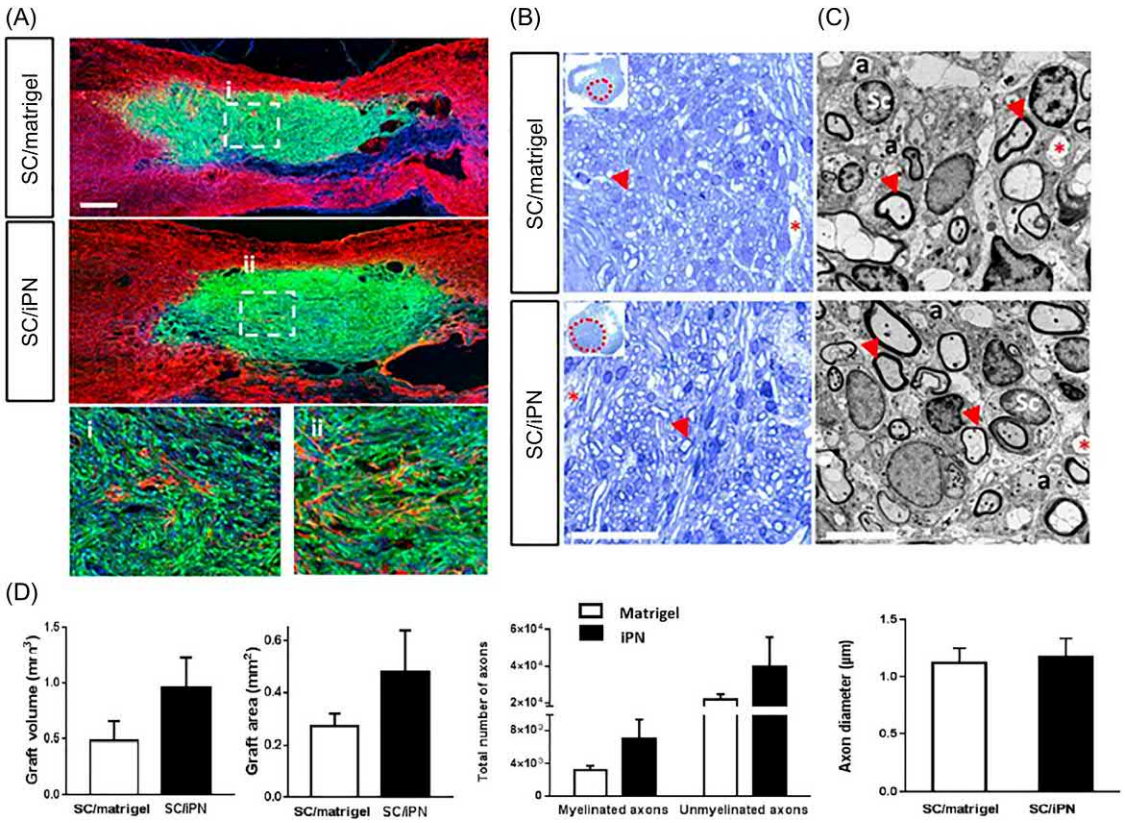

FIGURE 58.10 **Decellularized ECM: acellular iPN matrix.** (A) Sagittal spinal cord sections at 8 weeks post-transplantation of GFP-positive SCs in either Matrigel or iPN demonstrate that the engrafted cells occupy the contusion injury cavity, surrounded by GFAP-positive astrocytes (red). Higher magnifications of the central areas of SC grafts in Matrigel (i) or iPN (ii) are also shown. (B) Semithin, toluidine blue-stained transverse sections and (C) electron micrographs of the spinal cord injury site indicate axonal myelination by engrafted SCs (arrowheads) after 8 weeks in a typical peripheral nerve myelin pattern. Asterisks denote blood vessels. (D) Quantification of graft volumes and areas, and the number and diameter of axons trended toward improvements with iPN cell transplantation. *a*, Unmyelinated axons; *ECM*, extracellular matrix; *GFAP*, glial fibrillary acid protein; *GFP*, green fluorescent protein; *iPN*, injectable peripheral nerve; *SCs*, Schwann cells. *Adapted from Cerqueira SR, Lee YS, Cornelison RC, Mertz MW, Wachs RA, Schmidt CE, et al. Decellularized peripheral nerve supports Schwann cell transplants and axon growth following spinal cord injury. Biomaterials 2018;177:176–85, with permission.*

to agarose. It is a soft and immunologically inert material that has been used as an SCI biomaterial in similar ways. Alginate also is relatively poor in directly supporting axonal growth and requires modification. Used in the form of injectable gel systems and hydrogel disks, alginate is a highly effective matrix for incorporation of ECM peptides [163], controlled release of neurotrophic factors [164–166] or small molecules [167], and cell encapsulation [168–170], including gene-modified cells delivering therapeutic factors [171,172]. Freeze-dried sponges [173] and ice-templated linear conduits [174] releasing NT-3 and chABC [175] have been developed. Capillary hydrogel designs [176,177] (Fig. 58.12) have been used for macroarchitectural and anisotropic support for tissue regeneration in combination with ECM surface modification [178] and astrocytes, and as microchanneled scaffolds delivering MSCs, SCs, and cellular or viral delivery of BDNF [179,247] (Table 58.3).

The glycosaminoglycan chitosan is a carbohydrate polymer derived from the chemical deacetylation of chitin, a structural polysaccharide found abundantly in the shells of crustaceans, shellfish and insects. Depending upon the extent of the deacetylation, chitosan has an intrinsically positive surface charge which supports cellular adhesion onto the material surface, along with chemical modification of the surface [248]. Improvements in cell attachment to the polymer have been achieved by modifying the surface with ECM peptides [249,250]. A thermoresponsive polymer has also been produced with the addition of poly-L-lysine, and glycerol phosphate salts [251]. The use of chitosan for spinal cord tissue engineering has evolved from the bioengineering of single lumen conduits [180] and characterization of cellular interactions [181]. Early work demonstrated that chitosan conduits could support axonal regeneration and functional recovery after complete spinal cord transection [182]. When

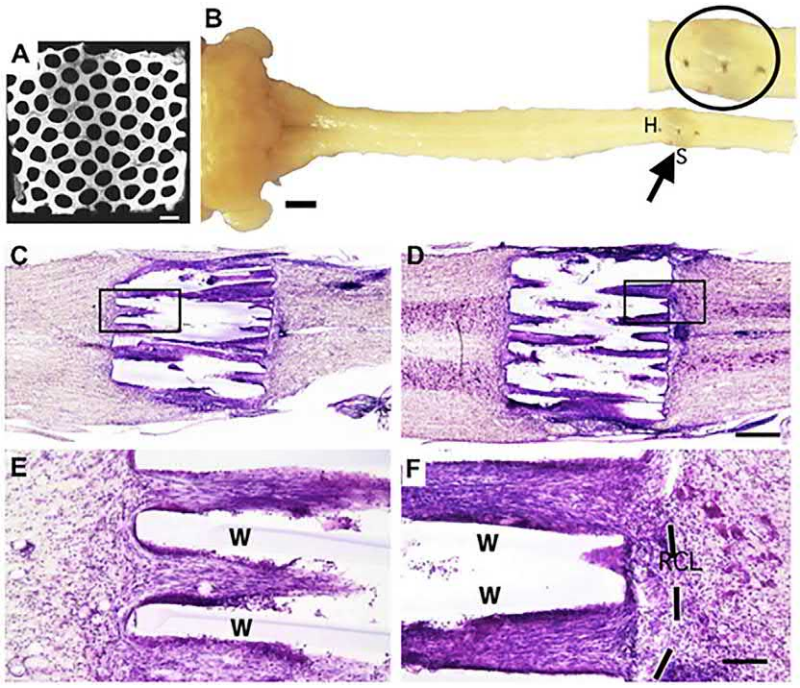

FIGURE 58.11 Agarose scaffolds. (A) SEM of a cross-section through a templated agarose scaffold details channel sizes of 200 μm and wall thicknesses of 66 μm. (B) Gross appearance of the scaffold implantation site with three dural sutures, 1 month after implantation into a complete spinal cord transection at the T3 level. (C–F) Nissl staining in longitudinal sections shows scaffold integration into the injury area with formation of regenerating tissue bridges, after either GFP-expressing (C and E) or BDNF-expressing (D and F) bone marrow stromal cells were loaded into the scaffold for implantation ("W" indicates scaffold wall). *BDNF*, Brain-derived neurotrophic factor; *SEM*, scanning electron microscopy. *From Gao M, Lu P, Bednark B, Lynam D, Conner JM, Sakamoto J, et al. Templated agarose scaffolds for the support of motor axon regeneration into sites of complete spinal cord transection. Biomaterials 2013;34(5):1529–36, with permission.*

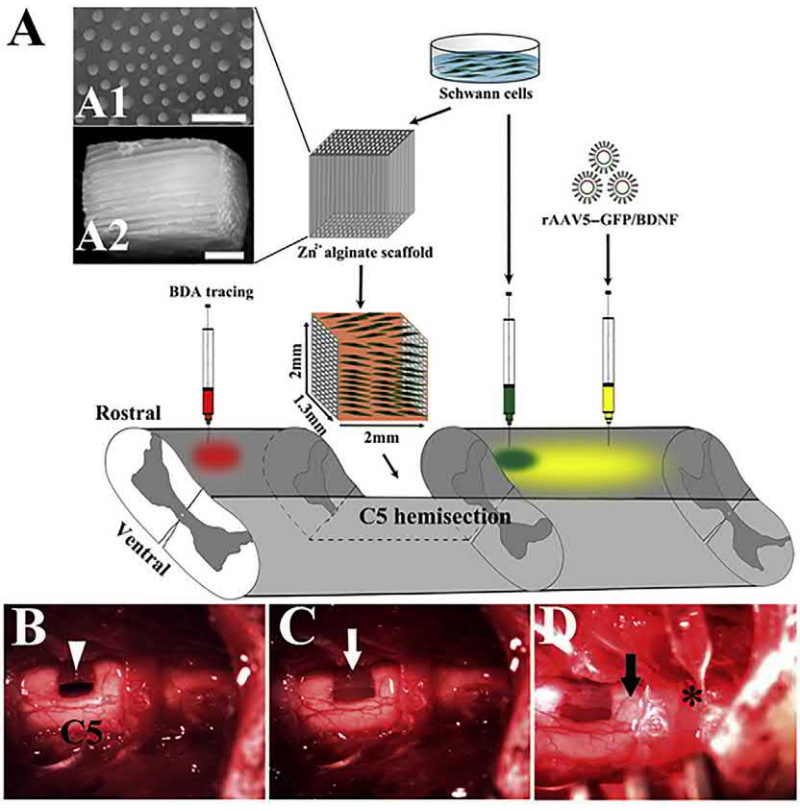

FIGURE 58.12 Alginate scaffolds. (A) A combinational approach is exemplified through the implantation of an SC-seeded alginate capillary hydrogel into a C5 dorsal hemisection injury. AAV5 serotype viral vectors (yellow) were injected into the adjacent caudal spinal cord for the expression of GFP or BDNF. Additional SCs (blue) were also injected into the adjacent cord. BDA (red) was injected rostrally for anterograde axonal tracing of descending axons. (B–D) A 2 mm hemisection (B) is grafted with the SC-loaded scaffold (C), and SCs and virus were injected into the spinal cord were indicated by the arrow and asterisk, respectively (D). *AAV*, adeno-associated virus; *BDA*, Biotinylated dextran amine; *BDNF*, brain-derived neurotrophic factor; *SCs*, Schwann cells. *Adapted from Liu S, Sandner B, Schackel T, Nicholson L, Chtarto A, Tenenbaum L, et al. Regulated viral BDNF delivery in combination with Schwann cells promotes axonal regeneration through capillary alginate hydrogels after spinal cord injury. Acta Biomater 2017;60:167–80, with permission.*

formulated as a topical gel, chitosan was shown to conform well to the shape of a transection injury [183,184] and to mediate secondary injury mechanisms, including suppression of reactive oxygen species [185]. Neural progenitor cells survived subcutaneous transplantation and differentiated within a chitosan gel [186], and MSCs were encapsulated and delivered into a complete transection for their paracrine activity and immunomodulation [63]. When chitosan was fabricated as a conduit, transplanted NSCs differentiated into astrocytic and oligodendrocyte lineages in the spinal cord to form regenerated tissue bridges [187,188]. These studies subsequently led to the key observation that a biomaterial could modify the distal and proximal stumps of a severe transection injury by inducing the alignment of endogenous radial glial cells for axonal guidance into the conduit [189].

Chitosan has proven to be a particularly versatile material for polymer-mediated delivery of molecular therapeutics, including modification as a carrier for PLGA microspheres eluting protein [190], and for neurotrophic factor elution to induce stem cell differentiation [191,192]. Recently, a chitosan conduit that eluted NT-3 provided for robust migration of endogenous neural progenitor cells from the adjacent cord into the lesion area, along with their differentiation into neural networks of ascending and descending tracts [193]. The findings of this key study, that endogenous neurogenesis could be elicited by the biomaterial, were then robustly validated in a second study, confirming anatomic bridging of neural tissue, locomotor recovery, and partial restoration of motor and sensory evoked potentials [194] (Fig. 58.13). Other investigators have used chitosan for exogenous neural progenitor cell delivery and differentiation aided in part by cyclic adenosine monophosphate (cAMP) elution from PLGA microspheres embedded within the conduit [186,195,196] (Fig. 58.14). Reformulations of chitosan as hydrogels and sponges have been successful in encapsulating MSCs as a source of paracrine trophic factor support [63,197] and as a reservoir of OPCs for axonal remyelination after injury [198]. A large animal primate study bridged a 1 cm thoracic cord hemitransection with an NT-3 eluting chitosan conduit, demonstrating motor and sensory functional recovery in addition to electrophysiologic and magnetic resonance imaging improvements with neural regeneration [199]. For molecular therapies, microglial activity has been targeted with small inhibitory RNA [200] and microRNA-based strategies [201] by eluting from chitosan nanoparticles.

Natural silk fibroin, including Tussah silkworm silk, *Antheraea pernyi* silkworm and *Bombyx mori* silkmoth fibers [252], have been used in biomaterial scaffolds for SCI repair as copolymer systems [202]. Silk fibers

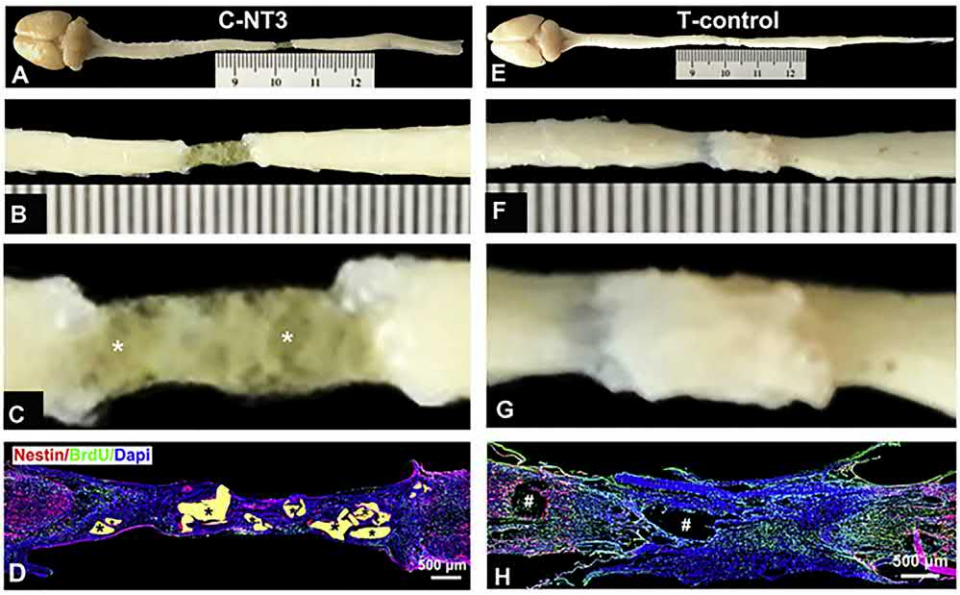

FIGURE 58.13 Validation study of NT-3 releasing chitosan scaffolds. Chitosan tubes filled with NT-3 releasing chitosan carriers were implanted into a 5 mm long segmental transection injury. Gross morphology demonstrated differing appearances of tissue cables after 3 months across the transection gap in animal receiving NT-3 scaffolds (A–D) and animals without implants (E–G). Longitudinal immunohistochemistry identified neural tissue with nestin, Tuj-1, and NeuN positive cells within the NT-3 supported tissue bridging. This key validation study indicated that NT-3 releasing scaffolds could facilitate neural regeneration by eliciting endogenous neurogenesis. Asterisks denote residual chitosan NT-3 carrier; number signs denote open cysts in control animals. *NT-3*, Neurotrophin-3. *From Oudega M, Hao P, Shang J, Haggerty AE, Wang Z, Sun J, et al. Validation study of neurotrophin-3-releasing chitosan facilitation of neural tissue generation in the severely injured adult rat spinal cord. Exp Neurol 2019;312:51–62, with permission.*

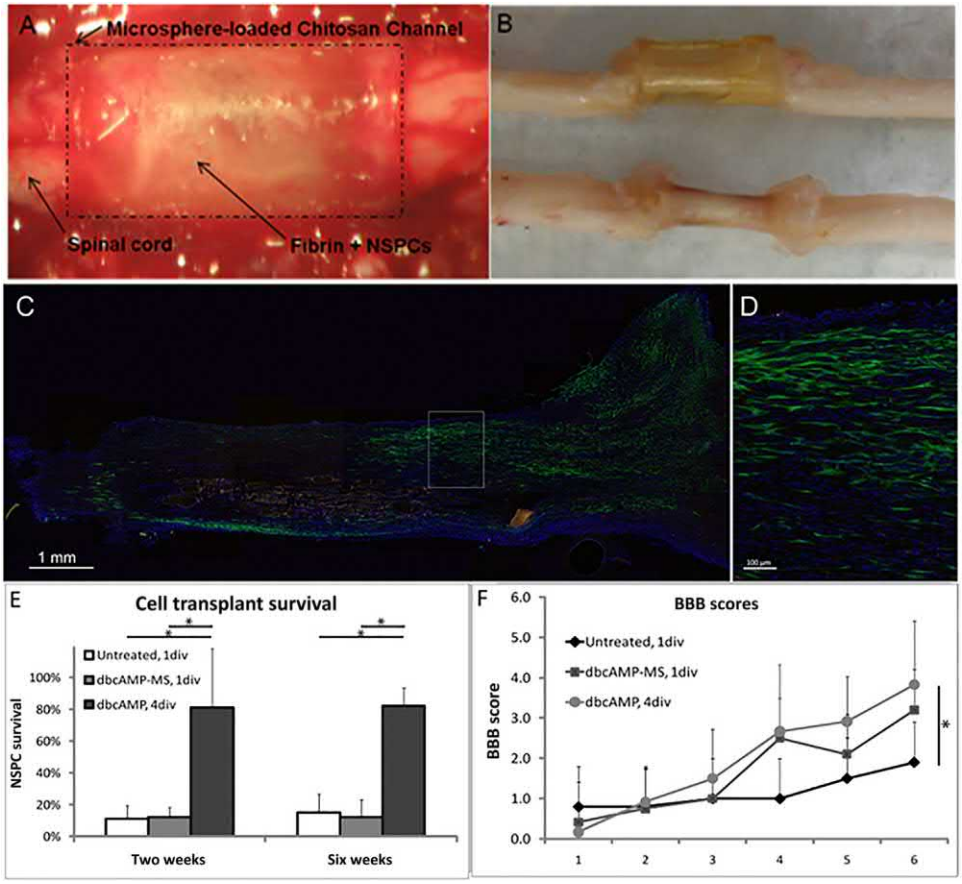

FIGURE 58.14 **Chitosan scaffolds and stem cell transplantation.** A chitosan conduit facilitated spinal cord tissue bridging, NSPC survival, and locomotor behavioral improvement over time. (A) Photographs of the surgical implantation of chitosan channels filled with fibrin and NSPCs also demonstrate (B) the formation of tissue bridges in completely transected animals 2 weeks after implantation. (C and D) Longitudinal section of the tissue bridge confirmed NSPC survival after 6 weeks in an animal receiving cells that were pretreated for 4 days with dbcAMP (dbcAMP, 4div). (E) NSPC survival after 2 and 6 weeks is represented for various treatment groups, comparing pretreatment with NSPCs transplanted in the presence of blank (untreated) or dbcAMP-releasing MS embedded into the scaffold wall after 1 day (1div) of incubation. (F) Assessment of functional recovery was performed according to the BBB locomotor scale. After 6 weeks, rats receiving transplants of dbcAMP-pretreated NSPCs show a statistically significant increases in hindlimb function relative to untreated animals. *BBB*, Basso, Beattie, and Bresnahan; *dbcAMP*, dibutyryl cyclic adenosine monophosphate; *MS*, microspheres; *NSPC*, neural stem/progenitor cell. *From Kim H, Zahir T, Tator CH, Shoichet MS. Effects of dibutyryl cyclic-AMP on survival and neuronal differentiation of neural stem/progenitor cells transplanted into spinal cord injured rats. PLoS One 2011;6(6):e21744, with permission.*

provide bioactive cues by means of structural tripeptide repeats. The use of silk in animal models of injury follows several years of in vitro characterization, to define the extent to which fibers could support longitudinal neurite outgrowth [253], and determine how cells might behave on the fiber surface [254]. The guidance of migrating olfactory ensheathing cells [255–257] and differentiation of stem cells [258] have been of particular interest. Silk may also be used by dissolving fibers in aqueous solution and conforming against a shaped surface for freeze–drying. Combinations of silk fibers with alginate microspheres for GDNF [203] or NGF release [204] by seeded MSCs enhanced the sparing of spinal cord tissue and improved the number of surviving neurons. Silk fiber matrices themselves can incorporate growth factors as they form in aqueous solution and may be applied as bioactive films onto the surfaces of other polymers. Sustained release of NT-3 from silk films lining a conduit of the synthetic polymer poly-ε-caprolactone (PCL) filled with NSCs yielded improved stem cell survival and rates of differentiation, axonal ingrowth, and functional outcomes after transplantation in a complete transection injury [205]. When recombinant analogs of the spider dragline silk spidroins were electrospun as a copolymer with PCL, the addition of neural progenitor cells promoted neural tissue ingrowth along parallel silk microfibrils, and neurogenesis from stem cell differentiation [206].

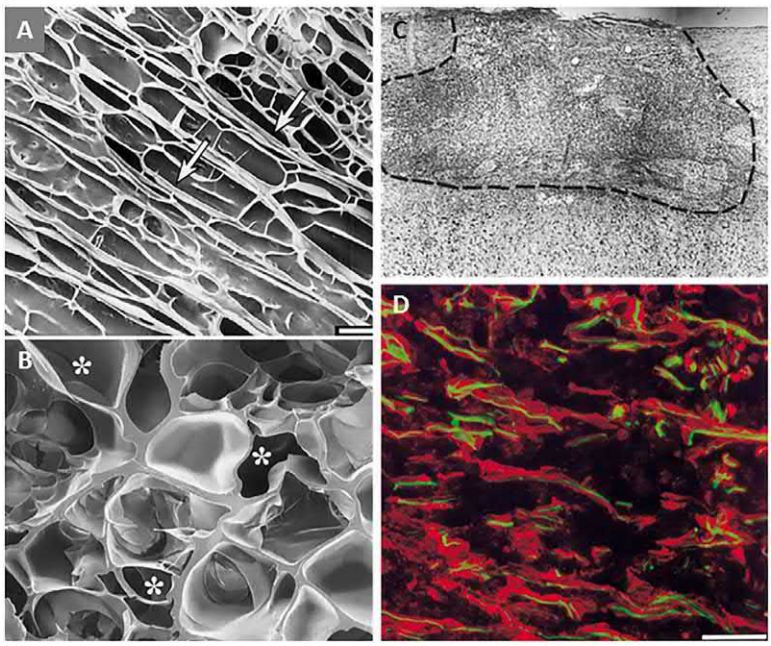

FIGURE 58.15 Fibronectin scaffolds. (A) SEM photomicrographs of a fibronectin mat detail longitudinally oriented strands of aggregated fibronectin (*arrow*). (B) In cross section, the strands form porous structures (*asterisks*) of approximately 20–100 μm in diameter. (C) Nissl staining of a fibronectin scaffold after 1 week post dorsal hemisection injury shows clean scaffold integration and cellular engraftment within the mat. (D) Axons (green, N-52 labeling) within the mat associated with p75-positive (red) SC processes. *SC*, Schwann cell; *SEM*, scanning electron microscopy. *Adapted from King VR, Henseler M, Brown RA, Priestley JV. Mats made from fibronectin support oriented growth of axons in the damaged spinal cord of the adult rat. Exp Neurol 2003;182 (2):383–98, with permission.*

Polymers derived from the blood

Biological matrices derived from blood, including frozen syngeneic peripheral blood, have been shown to support the formation of neural tissue, in addition to bone and cartilage tissues, when implanted in a complete spinal cord transection [259]. Fibronectin and fibrin, as two principle components of blood plasma matrices, are in use as spinal cord scaffolds. Their advantages include the ease of obtaining source materials as autologous grafts, as well as their inherent properties as fibrillary ECM proteins supporting cell adhesion, proliferation, and migration during provisional wound healing. Fibronectin mats have been produced with linearly aligned fibers to orient axonal outgrowth (Fig. 58.15), using embedded antibody therapies (transforming growth factor-β) [207] and sequestering neurotrophic factors (BDNF, NT-3) [208,209] within a porous architecture for slow release. Fibrinogen mat implantation supported axonal regeneration and deposition of laminin matrices around axons, the migration of endogenous SCs and OPCs with evidence of remyelination, and vascularization of the graft [210]. Additional benefits of neuroprotection were demonstrated with reductions in lesion size, rates of apoptosis in the adjacent cord, and secondary axonal injury following fibronectin mat placement into a hemisection injury [211]. Injectable formulations as fibronectin and fibrin copolymers also conformed well to laceration injury sites and supported axonal outgrowth [212].

Fibrin scaffolds are formed following the cleavage of fibrinogen by thrombin and cross-linking with Factor XIIIa [57,260]. The implantation of fibrin polymer scaffolds into a dorsal hemisection model delayed astrocytosis and gliosis of the adjacent cord, and improved neuron fiber extension into the graft [213]. The use of fibrin scaffolds as an affinity-based delivery system for molecular therapeutics in combinatorial approaches is a particularly powerful technology for spinal cord repair [261] (Fig. 58.16). Cross-linking heparin moieties [262] to fibrin through a Factor XIIIa-heparin bidomain peptide has allowed for the controlled release of noncovalently sequestered neurotrophic factors, including NT-3 [214,215], GDNF [216], and NGF [217,218]. The growth factors are slowly released via plasmin-mediated degradation of the fibrin scaffold by infiltrating cells. Codelivery of NT-3 from fibrin scaffolds improved neuronal fiber sprouting and reduced the extent of the adjacent glial scarring over controls in a complete transection model of injury [214,215]. Fibrin scaffolds have been used to direct embryonic stem cell–derived neural progenitor cell differentiation [219–221]. Controlled-release of trophic factors [222] enhanced transplanted stem cell survival and the efficiency of differentiation to neurons and other favorable cell types [223]. Additional stem cell enrichment using antibody selection to derive purer populations of progenitor motor neurons [224] yielded high rates of differentiation into neurons, oligodendrocyte, and astrocyte cell lineages when transplanted into a dorsal hemisection within an NT-3 eluting fibrin scaffold [225]. However, combination strategies with progenitor motor neurons and molecular approaches to reduce inhibitory factors in the cord, including controlled release of

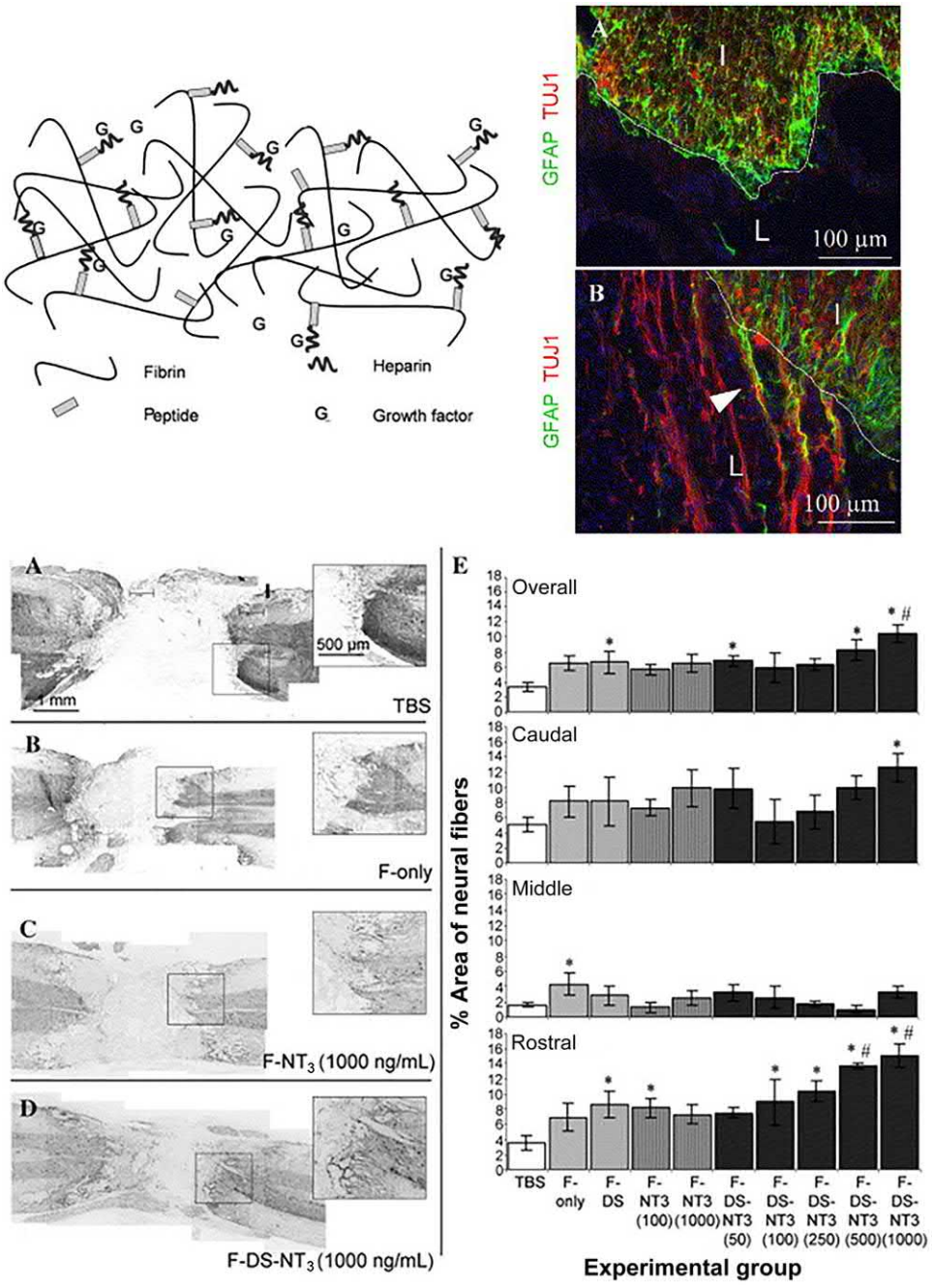

FIGURE 58.16 Fibrin scaffolds and the heparin binding delivery system. (Upper left panel) A schematic diagram of the heparin binding system details the covalent cross-linking of a bidomain peptide ($\alpha_2PI_{1-7}-ATIII_{121-134}$) with Factor XIIIa and electrostatic interactions with heparin at opposite ends of the molecule; heparin in turn sequesters a growth factor, which is slowly released upon degradation of the fibrin. (Upper right panel) Following complete spinal cord transection and placement of the fibrin scaffold alone (A) or with an NT-3 releasing system (B), neuronal fiber sprouting (red, Tuj1) (*arrowhead*) was observed to occur along an elongated astrocyte process (green, GFAP) in the NT-3 treated animal (*white dotted line*, lesion border). (Lower panels) Neuronal staining (Tuj1) (A–D) in transection only, fibrin only, fibrin with NT-3 dissolved at 1 μg/mL, and the heparin-fibrin DS with 1 μg/mL NT-3 demonstrated improved neuronal fiber sprouting with controlled release of NT-3 at higher loading doses, at various scaffold levels upon quantification (E). *DS*, Delivery system; *GFAP*, glial fibrillary acid protein; *I*, intact cord; *L*, lesion; *NT-3*, neurotrophin-3. *Adapted from Taylor SJ, Sakiyama-Elbert SE. Effect of controlled delivery of neurotrophin-3 from fibrin on spinal cord injury in a long term model. J Control Release 2006;116(2):204–10, with permission.*

ChABC and the NOGO receptor antagonist NEP1-40, appeared to increase macrophage infiltration into the graft and reduced stem cell survival [226]. Several highly promising approaches to select for other specific neuronal lineages from stem cells, including V3 [263] and V2a interneurons [264], could be combined with biomaterials for transplantation to reduce teratoma formation risks from undifferentiated cells, and promote improved differentiated cell survival and function in vivo [265].

Biomaterials for spinal cord tissue engineering: synthetic polymers

Compared to natural polymers, the use of synthetic polymers offers wider scope to design and control multiple characteristics of the biomaterial. Synthetic polymers may have varying degrees of biodegradability. Common biodegradable materials include those designed around polyesters of lactic and glycolic acid (PLA and PGA), or

hydrogels based on a polyethylene glycol (PEG) chemical structure. Hydrogels that are based upon methacrylates typically do not biodegrade well. PLA and PGA were among the first materials used in the field, in part because they had a long track record of established safety in other clinical applications, including as absorbable sutures, as skin grafting materials, and as conduits for peripheral nerve repair [266,267]. Rapid advances in the development of hydrogels, in their chemical and structural versatility, have produced softer materials which are more suitable to spinal cord implantation in their mechanical properties than more firm and less aqueous lactic and glycolic acid polyester materials. Synthetic polymers have wider scope than natural polymers for chemical functionalization, including surface gradients of ECM or integrated neurotrophic factor gradients, and surface charge modification for cell adhesion and controlled release. A wide range of novel mechanisms for drug delivery and provision of molecular or genetic therapies have been highlighted. Complex surface topographies have been produced to optimize macro- and microarchitecture features through sophisticated molding or lithography techniques.

Poly α-hydroxy acid polymers

PLA, PGA, and their copolymer PLGA are polyester linkages of lactic and glycolic acid, which are hydrolyzed in vivo to release lactide and glycolide. PLA was among the first biomaterials used for SCI repair, characterized in a seminal study in 1998 to be a resorbable material that was compatible with SCs and with the spinal cord [268]. A single-channeled PLA scaffold loaded with SCs was then used within a complete transection model [269], demonstrating that axons and a vascular bed could regenerate into the construct, despite some degree of structural instability of the scaffold itself. A freeze−drying technique was then used to fabricate longitudinally aligned pores from a PLA foam impregnated with BDNF for sustained release. BDNF did not improve a relatively low yield in axon numbers [270]. Further modifications were made to incorporate SCs into the foam that had been genetically engineered to secrete a bifunctional neurotrophic protein (D15A) with BDNF and NT-3 activity [271]. Axonal regeneration was demonstrated over 6 weeks, while few SCs survived after the first week of scaffold placement. More recently PLA scaffolds with highly refined porosity and nanofibrous surface topographies have been developed, promoting neurite extension along the microarchitecture after complete transection [272] (Table 58.4).

The use of PLA alone has been superseded by scaffolds fabricated from the copolymer PLGA, which remains among the most widely used materials for single and multichannel spinal cord scaffolds and for controlled release of biological and small molecule therapeutics [273]. Initial studies demonstrated that multichannel PLGA scaffolds supported robust axonal regeneration when seeded with SCs, initially without functional improvements [274] (Fig. 58.17). The in vitro biomechanical characteristics of bending, swelling, deformation, degradation, and permeability of varying PLA:PGA ratios were subsequently determined for PLGA spinal cord scaffolds implants [275]. Surgical techniques for implanting these scaffolds with and without rigid spine fixation were optimized following complete spinal cord transection [276], and it was shown through fast blue retrograde tracing studies that regenerating axons traversed the length of the scaffold and extended into the opposite spinal cord [277]. Unmodified SCs, transplanted in PLGA in association with recombinant NT-3 injection into the caudal cord, improved functional recovery after complete transection [278]. Seeding scaffolds with SCs within PLGA multichannel augmented the number of regenerating axons when compared directly to NSCs [279,280]. Additional studies have explored the impact of NSCs in large animal models [281], and stem cell differentiation in vivo on electrospun PLGA copolymer nanofiber conduits [282]. MSCs have also been delivered from PLGA conduits [283] and microfiber mats [197].

The rate at which these PLGA scaffolds will dissolve can be controlled by modifying the proportions of lactic-to-glycolic acid, with an 85:15 PLA to PGA degrading significantly more slowly than a 50:50 ratio. This property in particular has extended the use of PLGA from being a physical conduit toward its principle, contemporary use in nanosphere or microsphere technologies for controlled release of therapeutic molecules (Fig. 58.18). Micro- and nanospheres are produced by microemulsion techniques, where an aqueous solution containing the molecule intended for delivery is emulsified in an organic phase polymer solution to create spherical droplets, which are then extracted into another external aqueous phase [333] and dried. The size of the droplet is controlled by the emulsion agitation rate, the composition of the aqueous and organic phases, and the addition of surfactants to modify the surface tension between the phases [72]. Other techniques include freeze−drying of aerosolized droplets. The rate of drug delivery depends on the initial concentration within the sphere, and the size of the sphere for a given polymer and its degradation kinetics.

Drug-eluting nano- and microspheres been used to deliver neurotrophic factors, including NT-3 and GDNF [273,284], biomolecules to modulate the immune response and wound healing, including methylprednisolone and minocycline [285], flavopiridol [286], FGF-2 [287,288], and to promote angiogenesis [289]. The particles may be injected as a suspension into a contusion or

TABLE 58.4 Applications of synthetic polymer scaffolds in spinal cord injury repair.

Synthetic polymers	Polymer structure	Architecture	Functionalization	Animal model	Examples of applications for spinal cord repair
Biodegradable polymers					
PLA	Poly(D,L-lactic acid)				*PLA*
PGA	Poly(glycolic acid)	Conduit		CT	Single channel conduit [269]
PLGA	Poly(lactic-*co*-glycolic acid)	Conduit	BDNF	CT	Freeze-dried macroporous foam scaffold with incorporated BDNF [270]
		Conduit	SC, BDNF/NT-3	CT	Freeze-dried macroporous scaffold with BDNF-secreting SCs [271]
		Conduit		CT	Porous and nanofibrous multichanneled microarchitectures [272]
					PLGA
		Conduit	SC	CT	Multichannel scaffolds seeded with SCs [273–277]
		Conduit	NT-3, SC	CT	Recombinant NT-3 and SC transplantation [278]
		Conduit	NSC	CT, HS	Multichannel scaffolds seeded with NSCs [279–281]
		Conduit	NSC	CT	Electrospun PLGA–PEG composite nanofiber scaffolds with NCS [282]
		Conduit	MSC	HS	Human MSC delivery [283]
					PLGA and controlled release technologies
		Nanoparticles	NT-3, GDNF	C	Neurotrophin-eluting micro and nanospheres within scaffolds [273,284]
		Nanoparticles	Methylprednisolone	HS	Immunomodulatory drug delivery [285]
		Nanoparticles	Flavopiridol	HS	Immunomodulation and glial scar reduction [286]
		Nanoparticles	FGF-2, MSC	C	FGF-2 releasing nanoparticles with MSC transplantation [287]
		Microfibers	FGF-2	HS	FGF-2 sustained release from microfibers [288]
		Microspheres	VEGF, bFGF	C	Angiogenic microspheres [289]
		Conduit	Lentivirus	HS	Multichannel conduits with polysaccharides coating for lentiviral delivery [240]
		Film	NGF DNA	HS	Fibronectin coating with NGF DNA lipoplexes [132]
		Nanoparticles	VEGF gene delivery	CC	Lipid modified PLGA nanoparticles for nonviral gene delivery [290]
PCL	Poly-ε-caprolactone	Microfibers			Nanofiber spinning for axonal growth orientation [291]
		Sheets			Microgrooved topographies [76,292]
		Conduit	NSC, NT-3, BDNF	HS	NSC delivery with BDNF [293] and NT-3 overexpressing F3 cells [294]
		Conduit	iPSC, SC	CT	iPSC–NSC delivery with activated SC [295]
		Conduit		CT	Porous PCL microtubules [296]
PCLF	Poly-ε-caprolactone fumarate	Conduit	SC	CT	Multichannel scaffolds seeded with SCs [297]

(*Continued*)

TABLE 58.4 (Continued)

Synthetic polymers	Polymer structure	Architecture	Functionalization	Animal model	Examples of applications for spinal cord repair
Biodegradable hydrogels					
PEG	Polyethylene glycol	Gel		C	Immunoprotective sealant gel [298,299]
		Gel		CT	Injectable gel for reapposition of transected cord stumps [300–302]
		Gel	MSC	CT	Encapsulation of MSCs [303]
		Gel	Neurons		Solid phase three-dimensional neurite growth matrix [304]
		Gel	ECM peptide, NSC		NSC differentiation [305]
		Gel	NT-3, BDNF	CT	Injectable gel for NT-3 and BDNF delivery [306–308]
		Gel		C	Intravenous solution with magnesium [309,310]
OPF +	Oligo[poly(ethylene glycol)fumarate]	Sheet	Neurons, SC		In vitro support of neurite outgrowth and myelination [311,312]
		Conduit	SC, MSC	CT	Comparison of SC and MSC delivery [41,313]
		Conduit	SC	CT	Comparison SC delivery from PLGA and PCLF [314]
		Conduit	GDNF, SC	CT	Delivery of GDNF-overexpressing SCs [48]
		Conduit	SC, rapamycin	CT	Immunomodulation with rapamycin-eluting PLGA microspheres [66]
Nonbiodegradable hydrogels					
pHEMA	Poly(2-hydroxyethyl methacrylate)	Sponge		HS	pHEMA sponges [315]
		Conduit	NGF		Guidance channels releasing NGF [315,316]
		Conduit	BDNF	Cervical HS	BDNF delivery [317]
		Conduit			Guidance channels templated over PCL microfibers [318]
		Conduit	ECM peptides		Cell adhesion gradients in fiber-templated scaffolds [319]
		Sheet	ECM peptides		Copolymer with chitosan including cell adhesion peptides [250]
		Conduit	NGF, NT-3, poly(L-lysine)		Neurotrophic gradients and neurite adhesion [320]
		Conduit	charge modification	HS	Surface charge modification for cell adhesion and axonal growth [321,322]
		Conduit	ECM peptides	HS	ECM-peptide macroporous scaffolds [323]
		Conduit	ECM peptides, MSC	CT	MSC delivery with ECM-peptide macroporous scaffolds [324]
		Conduit	iPSC–NPC, ECM	BC	iPSC–NPC delivery in laminin coated, positively charged scaffolds [325]

(*Continued*)

TABLE 58.4 (Continued)

Synthetic polymers	Polymer structure	Architecture	Functionalization	Animal model	Examples of applications for spinal cord repair
pHEMA-MMA	pHEMA-*co*-methyl methacrylate	Conduit		CT	Multilayered macroporous guidance channels [326,327]
		Conduit	NGF		Sustained release of NGF from inner lumen layering or embedded microspheres [328]
		Conduit	ECM, NT-3, FGF-1	CT	Conduit with channels filled with ECM and soluble NTF [130]
		Conduit		CT	Coil or polymer channel reinforcement [329]
pHPMA	Poly[*N*-(2hydroxypropyl) methacrylamide]	Gel		CT	pHPMA colloid gel [330]
		Gel	ECM peptide	CT	Colloid gel with cell adhesion peptides [331] (Neurogel)
		Conduit		HS	pHPMA block hydrogel [332]

BC, Balloon compression; *BDNF*, brain-derived neurotrophic factor; *C*, contusion; *ChABC*, chondroitinase ABC; *CT*, complete transection; *ECM*, extracellular matrix; *FGF*, fibroblast growth factor; *GDNF*, glial cell-derived neurotrophic factor; *HS*, hemisection; *iPSC*, induced pluripotent stem cell; *MSC*, mesenchymal stromal cell; *NGF*, nerve growth factor; *NPC*, neural progenitor cell; *NSC*, neural stem cell; *NT-3*, neurotrophic factor-3; *SC*, Schwann cell; *VEGF*, vascular endothelial growth factor.

hemisection defect, or can be suspended in a scaffold of another material such as alginate [164] and chitosan [190]. The delivery of genetic therapies by viral [240] and nonviral PLGA composites are also being explored to transduce the local spinal cord tissue to overexpress neurotrophic factors and angiogenic factors [132,290] (Table 58.4).

PCL is a relatively stiff biodegradable material, favored for its ease of use in electrospinning techniques [291], and in microgrooved topographies in flat and rolled−spiral sheets [76,292] to longitudinally orient axonal extension. PCL has been shown to be more effective as a scaffold material after such modifications are made to the microarchitecture. PCL nanofibrous scaffolds incorporating immobilized BDNF have also been used in the setting of in vitro NSC differentiation [293], the delivery of NSCs into a hemisection model in association with NT-3 overexpressing cells [294], and for the delivery of SCs with iPSC−NSCs [295]. Additional functionalization of microtubules of PCL with salt-leaching for porosity promoted axonal extension throughout the open volume of the graft [296]. A multichanneled scaffold fabricated from the PCL derivative, poly-ε-caprolactone fumarate (PCLF), loaded with SCs, was shown to have a comparable regenerative capacity to PLGA scaffolds, but was inferior to a PEG-based hydrogel [314] (Table 58.4).

PEG is a highly versatile, water-soluble, linear hydrogel polymer that has been extensively used in SCI repair in a variety of chemical formulations [334]. The principle applications have included being applied directly as an injectable gel to a spinal cord defect as a sealant or surfactant [298,299] (Fig. 58.19). PEG in this form may aid in the physical fusion of severed axolemma [335,336], as well as prevent free-radical membrane lipid oxidative stress through mitochondrial protection, and reduce calcium influx-mediated excitotoxicity [337]. Locomotor and electrophysiologic recovery after complete transection has been demonstrated using PEG as a "fusogen" to reappose the ends of the defect in rat [300] and canine models [301] and to direct long-distance axonal regeneration through the grafted area [302]. PEG sealants have also been used to encapsulate cells, including MSCs within complete transections [303], to grow neurons [304] and to differentiate NSCs [305]. Injectable PEG gels have delivered neurotrophic factors, including BDNF and NT-3 [306−308], PEGylated pharmacologics, including ionic magnesium [309,310], ECM proteins as molecular gradients [305], or copolymer systems for in situ gelation [338] (Table 58.4).

Solid phase, porous PEG-based polymers for the delivery of cells and neurotrophic factors have also been developed [304,339]. As an example that highlights several properties of PEG hydrogel scaffolds, the positively charged PEG derivative oligo[poly(ethylene glycol)fumarate] (OPF +) is a photo-cross-linked soft, porous, biodegradable hydrogel with biomechanical properties similar to spinal cord tissue [311,312] (Fig. 58.20). Implantation of OPF + scaffolds with and without SCs in Matrigel reduced collagen scarring, cyst formation and proteoglycan accumulation at the injury interface to the same extent over transection injury alone [313], indicating that the presence of the PEG-based scaffold itself may reduce the

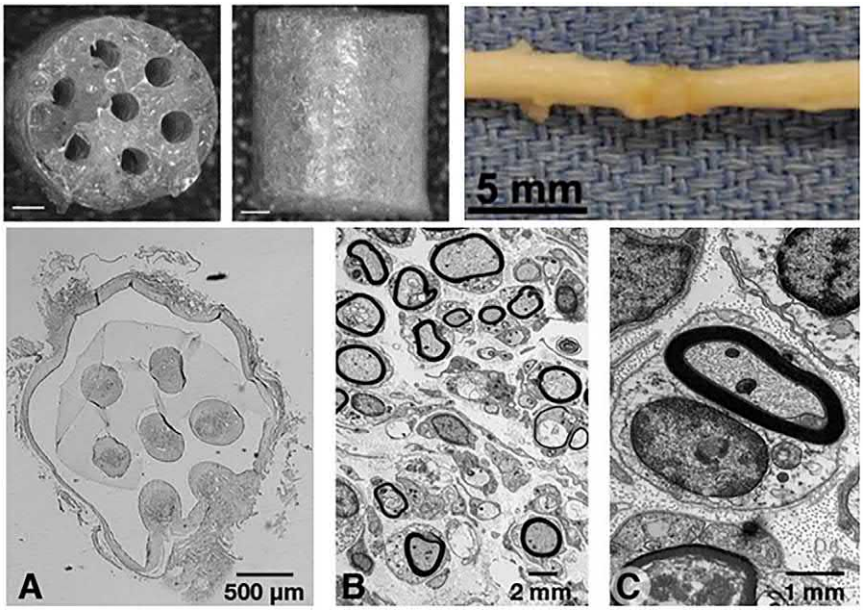

FIGURE 58.17 PLGA scaffolds. (Upper panels) A multichannel PLGA scaffold for placement into a complete transection in rats measures approximately 3 mm in diameter, 2 mm in length, and contains 7 parallel channels of 450 μm in diameter. The scaffold was loaded with Schwann cells, and integrated well between the free ends of spinal cord after 8 weeks. (Lower panels, A) Axons and blood vessels regenerated within a central core area within channels, which was circumferentially surrounded by more dense fibrotic tissue devoid of axons. Regeneration was observed both within and through the implant into the opposite cord tissue, as demonstrated by retrograde fast blue axonal tracing. (B and C) Schwann cells myelinated axons in a typical one-cell-to-one-axon ratio as seen by transmission electron microscopy, including extracellular deposition of collagen fibrils and basal lamina. *PLGA*, Poly(lactic-*co*-glycolic acid). *Adapted from Moore MJ, Friedman JA, Lewellyn EB, Mantila SM, Krych AJ, Ameenuddin S, et al. Multiple-channel scaffolds to promote spinal cord axon regeneration. Biomaterials 2006;27(3):419–29; (Lower panels) Reproduced with permission from Chen BK, Knight AM, de Ruiter GCW, Yaszemski MJ, Currier BL, Windebank AJ. Axon regeneration through scaffold into distal spinal cord after transection. J Neurotrauma 2009;26(10):1759–71; © Mary Ann Liebert, Inc. New Rochelle, NY. and Chen BK, Knight AM, Madigan NN, Gross L, Dadsetan M, Nesbitt JJ, et al. Comparison of polymer scaffolds in rat spinal cord: a step toward quantitative assessment of combinatorial approaches to spinal cord repair. Biomaterials 2011;32:8077–8086, with permission.*

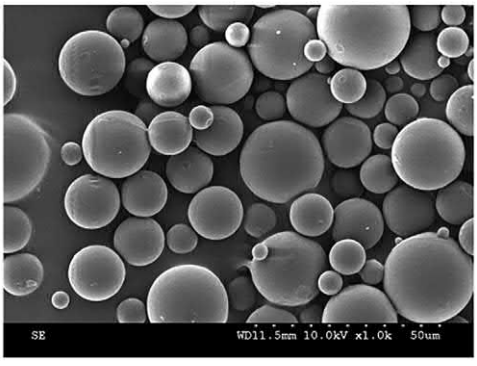

FIGURE 58.18 PLGA microspheres and nanoparticles. SEM photomicrograph at 1000× showing the ultrastructure of nanoparticles fabricated from PLGA using a double emulsion technique for the polymeric delivery of a small molecule, flavopiridol, a nonselective cyclin D kinase/cell cycle inhibitor. *PLGA*, poly(lactic-*co*-glycolic acid); *SEM*, scanning electron microscopy. *Adapted from Ren H, Han M, Zhou J, Zheng ZF, Lu P, Wang JJ, et al. Repair of spinal cord injury by inhibition of astrocyte growth and inflammatory factor synthesis through local delivery of flavopiridol in PLGA nanoparticles. Biomaterials 2014;35 (24):6585–94, with permission.*

formation of barriers to regeneration. Astrocyte infiltration and glial scarring were also reduced, consistent with the observations of others [340]. OPF+ scaffolds loaded with primary SCs improved axonal regeneration density over transplanted MSCs [41], and the accuracy of growth orientation when systematically compared to other SC-loaded polymer constructs [314] including PLGA and PCLF. OPF+ hydrogel scaffolds have also been designed to incorporated the antifibrotic drug rapamycin within PLGA microspheres, which reduced the foreign body inflammatory response and scar tissue formation and promoted partial functional recovery after 6 weeks [66]. SCs that have been genetically modified with retrovirus to secrete a high concentration of GDNF in hydrogel scaffolds further enhanced axonal regeneration, remyelination, and promoted the outgrowth of ascending intraspinal motor neurons through the polymer implant [48] (Table 58.4).

Nonbiodegradable hydrogels

Poly(2-hydroxyethyl methacrylate) (pHEMA) is a nonbiodegradable hydrogel used to fabricate soft contact lenses, and therefore has advantages in spinal cord repair

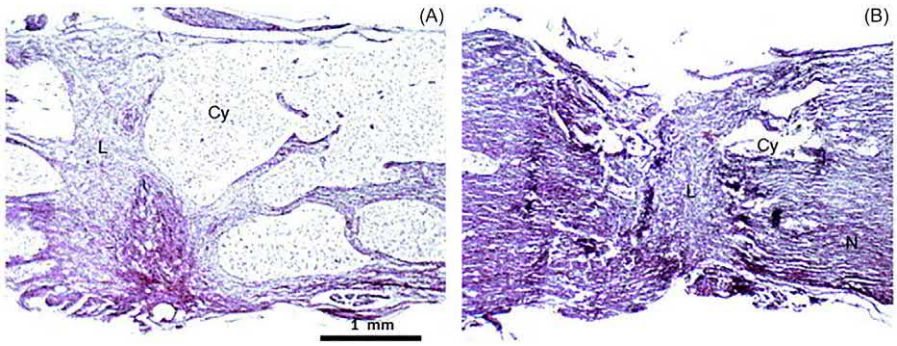

FIGURE 58.19 PEG as a fusogen. Thoracic spinal cord sections, stained with Holme's silver stain and counterstained with neutral red, 1 month after forceps compression injuries (A) without and (B) with topical application of PEG (M_r 1800, 50% w/v in water). Extensive areas of axonal loss and cystic cavitation in control animals are significantly reduced by fusogen treatment. *Cy*, cyst; *L*, lesion; *N*, intact spinal cord parenchyma; *PEG*, polyethylene glycol. *Adapted from Duerstock BS, Borgens RB. Three-dimensional morphometry of spinal cord injury following polyethylene glycol treatment. J Exp Biol 2002;205(Pt 1):13–24, with permission.*

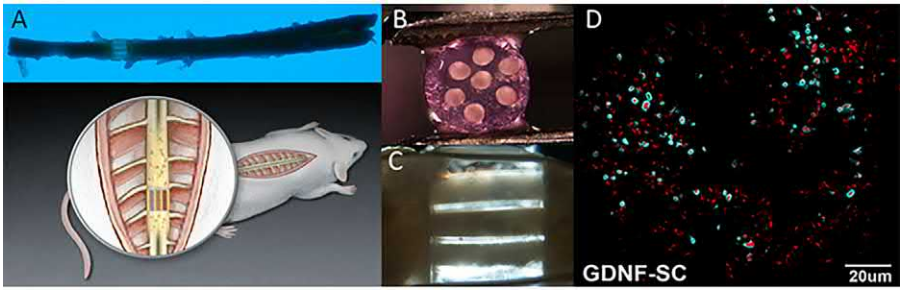

FIGURE 58.20 Positively charged OPF+. (A) A multichannel OPF+ hydrogel scaffold, loaded with SCs (B), implanted into a complete transection SCI at the T9 spinal level in rats. (C) Regenerating tissue bridges containing axons are seen to traverse the scaffold channels within the soft, translucent hydrogel. (D) In a GDNF–SC-loaded scaffold channel, approximately 250 regenerating axons are identified by immunostaining with βIII-tubulin (red), a proportion of which have been myelinated by SCs and are surrounded by myelin basic protein (cyan). GDNF–SCs enhanced the number of regenerating axons and the rate of myelination axons over that seen with unmodified SCs. *GDNF*, Glial cell-derived neurotrophic factor; *OPF+*, oligo[poly(ethylene glycol)fumarate]; *SCI*, spinal cord injury; *SCs*, Schwann cells. *(C) Reproduced with permission from Madigan NN, Chen BK, Knight AM, Rooney GE, Sweeney E, Kinnavane L, et al. Comparison of cellular architecture, axonal growth, and blood vessel formation through cell-loaded polymer scaffolds in the transected rat spinal cord. Tissue Eng, A 2014;20(21–22):2985–97; © Mary Ann Liebert, Inc. New Rochelle, NY. and Chen BK, Madigan NN, Hakim JS, Dadsetan M, McMahon SS, Yaszemski MJ, et al. GDNF Schwann cells in hydrogel scaffolds promote regional axon regeneration, remyelination and functional improvement after spinal cord transection in rats. J Tissue Eng Regen Med 2018;12(1): e398–407, with permission.*

applications as a clinically-approved polymer. pHEMA and pHEMA-*co*-methyl methacrylate (pHEMA-MMA) have been extensively developed for use as spinal cord scaffolds. Early applications as pHEMA sponges placed into dorsal hemisections [315] soon evolved into multilayered guidance channels [316] which immobilized NGF [341] and BDNF [317] for sustained release and which had longitudinally oriented channels of tunable size created by templating the pHEMA over PCL microfibers [318]. Templated conduits were subsequently functionalized with ECM peptide gradients in a pHEMA copolymer system with 2-aminoethyl methacrylate [319] and in a methacrylamide-chitosan composite [250]. A variety of surface modifications improve cell adherence and axonal extension, including incorporation of poly(L-lysine) and gradients of NT-3 [320]. Microfluidic techniques were employed to create functional concentration gradients by casting the scaffold using two or more inlet ports for differential mixing coupled with rapid polymerization.

Modification of surface charge, with the addition of quaternary amine groups or of a second methacrylate subtype [321], is another approach taken to improve cell/neurite migration and encapsulation, as many cell types adhere better to a positively charged surface. Macroporous pHEMA scaffolds [322] were evaluated in a dorsal hemisection injury bridged with four pHEMA composites, demonstrating an improvement in axonal regeneration into the core of the scaffold and a reduction in astrocyte infiltration in the positively charged scaffold. Cell surface interactions in longitudinally

oriented macroporous scaffolds were further modified using pHEMA copolymers with cholesterol methacrylate and ethylene dimethacrylate [342], or with ECM peptides [323], to successfully bridge a hemisection cavity with regenerated neural tissue. ECM peptide scaffolds were subsequently loaded with MSCs in a complete transection model, with MSCs not improving the degree of tissue and axon infiltration [324]. Recently, positively charged pHEMA scaffolds, coated with laminin and loaded with iPSC-derived NPCs in a balloon compression model reduced the degree of cavitation, supported axonal sprouting and stem cell survival [325] (Table 58.4).

pHEMA-MMA formulations of conduits were produced using a liquid–liquid centrifugation casting technique to produce a gel-like outer layer and a macroporous inner layer [326] (Fig. 58.21). Long-distance axonal regeneration through a T8 thoracic transection was demonstrated, with retrograde tracing studies identifying several brainstem nuclei to be the neuronal source of some axons traversing the graft [327]. A similar casting technique was employed for sustained release of NGF from inner lumen coatings or embedded microspheres [328]. Further improvements in supraspinal neuronal regeneration were achieved by filling the conduit channels with extracellular matrices and soluble trophic factors (FGF-1 and NT-3). Structural instability of the channel architecture was addressed by coil reinforcement and polymer with greater elastic moduli [329], but densities of axons did not improve, and the more rigid scaffold lead to

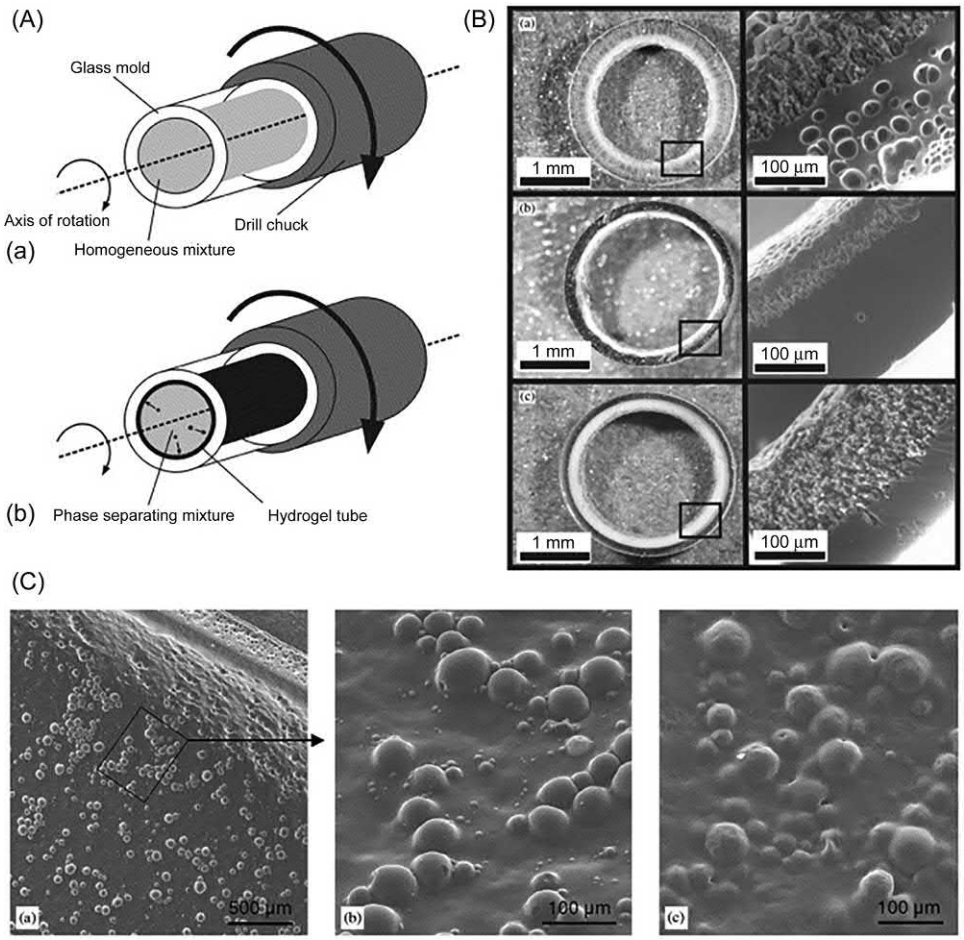

FIGURE 58.21 pHEMA tubes as copolymers with MMA. (A) Liquid–liquid phase separations in spinal cord conduit manufacture are produced by rotational force, (B) creating multiple layers of varying polymer porosity which dependent upon the concentration of MMA added to the pHEMA monomer mixture [in (B), 25% monomer that includes (a) 5% MMA, (b) 7% MMA, and (c) 10% MMA]. (C) NGF releasing PLGA microspheres were cast against the inner surface of the conduit lumen by a similar method, using pHEMA-MMA channels coated with an inner layer of pHEMA and 1 wt.% PLGA 85/15 microspheres: (a) at 40× magnification for a 5 wt.% pHEMA layer; (b) a close-up of (a) at 220× magnification for a 5 wt.% pHEMA layer; and (c) at 200× magnification for a 10 wt.% pHEMA layer by SEM. *MMA*, Methyl methacrylate; *NGF*, nerve growth factor; *pHEMA*, poly(2-hydroxyethyl methacrylate); *PLGA*, poly(lactic-*co*-glycolic acid); *SEM*, scanning electron microscopy. *Adapted from Dalton PD, Flynn L, Shoichet MS. Manufacture of poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) hydrogel tubes for use as nerve guidance channels. Biomaterials 2002;23(18):3843–51 (A and B) and Piotrowicz A, Shoichet MS. Nerve guidance channels as drug delivery vehicles. Biomaterials 2006;27(9):2018–27 (C), with permission.*

the development of syringomyelia and caudal migration of the rostral stump in some transected animals (Table 58.4).

Poly[*N*-(2hydroxypropyl)methacrylamide] (pHPMA) represents an additional methacrylate based polymer with more limited use for SCI repair. Early work demonstrated the capacity of a colloid pHPMA gel, with [330] or without [331] the incorporation of ECM peptide, to support regeneration when injected into a complete transection defect. When formulated as a hydrogel block, insertion of pHPMA into a thoracic hemisection improved animal locomotor scores and electrophysiologic parameters over controls in addition to supporting axonal regeneration within the hydrogel matrix [332] (Table 58.4).

Conclusion and future directions: the promise of clinical translation

SCI remains a devastating medical condition, with one-third of patients never to recover any neurologic function below a complete injury, and two-thirds of patient living with severe debility. With no effective clinical treatments beyond the acute phase of injury, tissue engineering to regenerate functional spinal cord tissue is poised to be a therapeutic intervention in the subacute to chronic phases. Remarkable progress over the last 20 years has been made using biomaterials to restore spinal cord neural tissue and to improve motor, sensory and autonomic nerve function in animal models, progress which has accelerated over the last 10 years. Engineering principles of material compatibility, macro- and microengineering strategies, and the ability for bridging biomaterials to deliver cellular and molecular therapeutics in combinations, have begun to effectively address the complexity of the injury process as a physical, cellular, and molecular continuum of barriers. This chapter has highlighted current strategies in a range of natural and synthetic polymers and their functionalization.

The ultimate goal of tissue engineering for spinal cord repair is to develop patient-specific therapies to facilitate functional recovery in injured people. Current Good Manufacturing Practices (cGMP) protocols and Investigational New Device and Device Exception applications within the United States Department of Health and Human Services Food and Drug Administration (FDA) are appropriately robust to ensure scientific rigor and patient safety. They are also compatible with the development of first-in-human trials along defined regulatory pathways [343]. Currently, an FDA-approved, multicenter clinical trial (NCT02138110, The INSPIRE Study) is evaluating the safety of implanting a PLGA biomaterial scaffold into spinal cords of patients with complete thoracic (AIS-A) SCI [344]. This study in turn will have implications for additional scaffold-based clinical trials with other polymer types and with biomaterial, cellular, and molecular therapeutics. Scaffolds in combination with autologous transplantation of patient derived SCs may be the logical next step, given the pioneering work of investigators at the Miami Project to Cure Paralysis to establish cGMP standard operating procedures for SC isolation from patient nerve biopsy specimens. The feasibility and safety of using autologous human SCs in Phase I (NCT01739023, NCT02354625) clinical trials for subacute and chronic SCI has been demonstrated [345], paving the way for the first-in-human combinatorial biomaterial trials for SCI.

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Chapter 59

Protection and repair of hearing

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Introduction

Deafness is the United States' primary disability, with hearing loss affecting more than 35 million individuals. The cause of hearing loss can generally be divided into two major categories: genetic hearing loss and acquired hearing loss. Genetic hearing loss is commonly separated into syndromic and nonsyndromic hereditary hearing loss [1−3], while acquired hearing loss can be caused by numerous etiologies accumulated over a lifetime, such as exposure to excessive noise, the use of ototoxic medications, bacterial or viral ear infections, head injuries, and the aging process. Importantly, these effects may coexist and thereby confound investigations in both animal models and humans. With our increasing aging population, age-related hearing loss (ARHL, presbyacusis) becomes a common problem in the elderly population, affecting one in three individuals over the age of 65 and 50% of individuals over the age of 75.

An improved understanding of the mechanisms underlying genetic, acquired, and "mixed" deafness has driven the development of new interventions and treatments as well as the refinement of previous approaches to prevent hearing loss and restore hearing after deafness. Hearing loss induced by cochlear damage from overstimulation, ototoxic drugs, trauma, infections, and/or aging can be reduced by reducing oxidative stress to the cochlea with antioxidants and enhancing endogenous protective systems with agents such as neurotrophic factors and HSPs. Treatment with antiinflammatory agents can also reduce acquired hearing loss. Genetic modification has now been successful in animal models and may become clinically feasible, with development of safe and effective vectors to treat genetic hearing loss as well as induce regeneration of hair cells. Placement of exogenous stem cells and transdifferentiation of endogenous cells is being developed as a strategy to replace lost hair cells or auditory nerve (AN) and to supplement remaining function. Survival and growth factors can be applied to the cochlea to improve AN survival following deafness and to induce the regeneration of its peripheral processes. Major advances have also been made in region specific delivery of therapeutics to the cochlea to allow these interventions, including gene transfer, microcannulation, and biomaterials placed into the middle ear or directly into the cochlea. Auditory prostheses are being refined, with significant improvements occurring in cochlear prostheses as well as in development of central auditory prostheses. Prostheses can also be engineering for application of therapeutics through microchannels. Biomaterial on prostheses can not only improve function and histocompatibility but also provide a means for delivering therapeutics. Artificial cochleae capable of transforming sound-induced movement of cochlear fluids into electrical signals are also being developed. The strategies for tissue engineering interventions based on recent scientific discoveries are the focus of this chapter. The chapter will examine interventions for protection from acquired sensory cell hair cell loss as well as loss of sensory cell—AN connections as well as for genetic hearing loss. It will then examine interventions for repair and replacement.

Protection from "acquired" sensory hair cell loss

While there may be some common mechanisms underlying the loss of sensory cells from noise, drugs, aging, and other cause of acquired deafness, there may also be

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differences. The following section will also focus on the underlying mechanisms that have given rise to therapeutic interventions against noise-induced and drug-induced hearing loss.

Oxidative stress and stress-related mitochondrial pathways

Oxidative stress, the overproduction of reactive oxygen species (ROS; free radicals) in the cell, has now emerged as a common mechanism for many traumatic insults, including noise and ototoxic drugs. In an unchallenged cell, ROS are products and by-products of normal metabolism and contained within a physiological range (redox homeostasis) by endogenous antioxidant systems. However, an excess of ROS will cause cellular damage, ultimately triggering cell death pathways of apoptosis and necrosis. The formation of ROS, such as superoxide, hydroxyl radical, and nitric oxide, may occur by different mechanisms depending upon the stress, but in each instance it appears early and continues throughout the traumatizing events. ROS can be formed by nonenzymatic reactions via redox-active iron complexes of drugs [4,5] and by enzymatic reactions in the cell. On the cellular level, oxidative stress may originate in the mitochondria [6,7] or may be due to the activation of NADPH oxidase via Rho-GTPases, a primary source of superoxide radicals [8–10]. Furthermore, overproduction of ROS may result in transient cellular energy depletion as documented by increased p-AMPKa in sensory hair cells [8,9,11]. Because a cellular redox-imbalance can be influenced by external manipulations, ROS-mediated hearing loss is a potential target for interventions to prevent hearing loss.

Calcium influx

Dysregulation of cytosolic calcium homeostasis is involved in noise- and aminoglycoside-induced hearing loss. Noise exposure increases free Ca^{2+} in outer hair cells (OHCs) immediately after acoustic overstimulation, mainly through mechanoelectrical transduction channels, but liberation from intracellular stores might also contribute [12]. Calcium influx appears to be associated with exposure at higher noise intensities. Guinea pigs exposed to 110-dB sound pressure level (SPL) noise displayed increased free calcium in isolated OHCs followed by OHC death and hearing loss, while a nonpathologic sound level of 75-dB SPL did not alter intracellular calcium levels [13]. Further evidence of modulation of intracellular Ca^{2+} levels after noise exposure can be deduced from an increase in the Ca^{2+}-binding protein calmodulin (CaM), a critical mediator of calcium signaling [13]. In addition, influx may be linked to increased endolymphatic calcium levels, which generates a high concentration gradient, driving calcium into sensory cells through voltage-gated calcium channels (VGCCs), particularly in inner hair cells (IHCs) [14]. These elevated calcium levels may contribute to sensory hair cell death via triggering of mitochondria-mediated caspase-dependent cell death pathways in sensory hair cells [15,16]. Furthermore, the mitochondrial calcium uniporter (MCU), which is a specific calcium channel in the mitochondrial inner membrane, affects the cell sensitivity to apoptosis through regulation of calcium uptake [17,18]. An elevation of MCU and reduction of Na^{+}/Ca^{2+} exchanger (NCLX) (sodium calcium exchanger), which extrudes calcium from mitochondria, leads to calcium overload in mitochondria, and initiates the loss of IHC synaptic ribbons and OHCs during noise exposure [19]. As another potential mechanism, calcium overload in IHCs can stimulate the excessive release of the neurotransmitter glutamate. The overactivation of glutamate receptors then causes excitotoxicity. Finally, the endoplasmic reticulum (ER) is a major subcellular compartment for intracellular calcium stores, and ER stress–mediated apoptosis has been associated with noise-induced, ototoxic drug-induced hearing loss, or ARHL [20–23].

Endoplasmic reticulum stress

In addition to being an intracellular calcium store the ER is involved in the process of protein folding and transport. Perturbations in protein-folding capacity caused by hypoxia, starvation, or ROS lead to ER stress [24–27]. When the stress response is initiated, cells can reduce the synthesis of general proteins through the activation of the unfolded protein response and induce the formation of intracellular synthetic molecular chaperones to restore proteostasis [28–30]. When the stress overwhelms the protective capacity of the unfolded protein response (UPR), the accumulation of unfolded or misfolded proteins can result in induction of ER-related apoptotic pathways. Under intense noise exposure the UPR does not completely eliminate misfolded proteins, ultimately activating ER-related apoptosis [31]. The expression of ER-related proapoptotic factor C/EBP homologous protein is increased in the cochleae of aged mice, indicating that ARHL may be related to ER stress [22].

Prevention of ototoxicity

Aminoglycoside antibiotics, including neomycin, kanamycin, tobramycin, amikacin, gentamicin, and the chemotherapy anticancer agent cisplatin (CDDP), are the most commonly used drugs that cause the loss of hair cells and consequently of hearing (ototoxicity). Although this review will emphasize the cochlear effects of ototoxic agents, we should note that aminoglycosides can also

cause vestibular toxicity resulting in the loss of balance. In accordance with the stress responses outlined above, ROS increase significantly in inner ear tissues following aminoglycoside treatment both in organ explants and in vivo [4]. Conversely, cellular antioxidant defense system constituents, such as glutathione, decrease after aminoglycoside treatment [32].

The redox imbalance resulting from increased ROS production and decreased antioxidant function then initiates competing signaling pathways of cell death and survival, involving intracellular organelles such as mitochondria and lysosomes and a network of interfacing signaling systems based on enzymatic actions (e.g., activation of proteases) and changes in gene expression (e.g., via transcription factors or epigenetic modifications). Indicative of this complexity, cell death from aminoglycosides includes both apoptosis and necrosis of hair cells in cochlear and vestibular organs [5,33,34]; and both caspase-dependent and -independent pathways appear to contribute to hair cell pathology [35,36]. The proapoptotic mediator c-Jun NH2-terminal kinase (JNK) is often involved, since pharmacological inhibitors (e.g., CEP-1347) can offer some protection in vitro from aminoglycoside damage [37,38]. Like the caspase pathway, involvement of the JNK pathway is reported mostly in cultured explants but may contribute to the overall pattern of cell death in vivo. A dominance of caspase-independent cell death emerges in a chronic kanamycin-treated mouse model, where the onset of cochlear functional deficits is delayed and continues to develop after the cessation of treatment, akin to the clinical situation. In this model, activated calpain and cathepsins are the major mediators of cell death [10]. Impacting a different pathway to cell death, the autophagy activator rapamycin prevents CDDP and gentamicin-induced hair cell apoptotic processes through the induction of autophagy and elimination of oxidative stress, while autophagy inhibitor 3-methyladenine (3-MA) accelerates hair cell apoptosis via the suppression of autophagy [39,40]. More recently, inhibitors of histone deacetylation have emerged as promising candidates for intervention. Histone deacetylase (HDAC) inhibitors, trichostatin and butyrate, can reduce gentamicin ototoxicity in explants by inhibiting the epigenetic histone modifications induced by aminoglycoside treatment [41]. Furthermore, the FDA-approved HDAC inhibitor vorinostat (SAHA) attenuates gentamicin-induced hair cell loss in explants [42] and in an acute mouse model in vivo [43]. However, it is not effective in chronic in vivo ototoxicity models using gentamicin in guinea pigs or kanamycin in mice [42], underscoring the complexity of translating preventive intervention from in vitro to in vivo models, let alone from animal models to the clinic.

Nevertheless, understanding of the mechanisms underlying both apoptotic and necrotic cell death in ototoxicity is the best basis for rational approaches to prevention. For example, small synthetic molecules designed to inhibit one of the many steps in the apoptotic cascade can potentially stave off cell death. For a clinical application, however, a systemic application of such powerful signaling molecules may have far-ranging physiological consequences, particularly when applied to antibiotic treatment, which may last for weeks or, in the case of tuberculosis, for months. Local gene therapy, the process of virally introducing a potentially protective gene locally into a tissue, may be more suitable in such a situation [44]. However, given the complexity of cell death mechanisms, targeting a single pathway may not be sufficient, as the inhibition of one might be bypassed by the activity of others. Furthermore, susceptibility to ototoxicity can be influenced by factors such as diet [32,45] or genetic predispositions, such as the mitochondrial mutations that can render people hypersensitive to aminoglycosides [46].

Currently, the most applicable method of prevention is antioxidant therapy, which has become a successful clinical approach for pathologies that involve free radicals. This type of intervention would act directly on the ROS upstream of the ensuing cell death pathways and therefore suppress toxic mechanisms at the very onset. A wide variety of antioxidant molecules have been shown to attenuate ototoxicity in vivo, constituting the most compelling support for ROS as major mediators of aminoglycoside-induced hearing loss [47–49]. Further support for the ROS is found in the mitochondrial peroxiredoxin 3 (Prx3); the radical scavenger *p*-phenylenediamine attenuates gentamicin-induced hair cell death through maintenance of Prx3 levels [7]. The attenuation achieved with various antioxidant treatments in animal models can be dramatic. For example, a gentamicin-induced hearing loss of 60–80 dB could be reduced to a negligible loss of 10 dB or less [47]. Since neither the serum levels of the drugs nor their antibacterial efficacy are compromised, antioxidant therapy provides a promising approach for clinical application.

A clinically feasible prophylactic therapy requires drugs that by themselves are nontoxic and easily administered to patients. One such example that emerged from laboratory studies is salicylate, the active ingredient of aspirin [50]. The efficacy of aspirin was tested in a randomized double-blind and placebo-controlled study in patients receiving gentamicin for acute infections [51]. Fourteen of 106 patients (13%) met the criteria of hearing loss in the placebo group, while only 3 out of 89 (3%) sustained a hearing loss in the aspirin group, for a 75% reduction in the incidence of ototoxicity. Aspirin did not influence the course of therapy. The protection from gentamicin-induced hearing loss with aspirin was confirmed by a second clinical trial [52]. Although aspirin is widely used in the United States as daily prophylaxis

against myocardial infarction, as well as for treatment of inflammation, fever, and pain, aspirin carries with it a small risk of reversible gastrointestinal side effects. Other antioxidants utilized in clinical trials to date include vitamin E and *N*-acetylcysteine (NAC). NAC reduced hearing losses in a small study of patients receiving gentamicin for bacteremia and in a study of peritonitis patients receiving aminoglycosides for peritoneal dialysis [53,54]. In contrast and despite success in animal studies, vitamin E did not reduce hearing loss in a small clinical study, underscoring the complexity of aminoglycoside responses in humans and the potential pitfalls of translation of animal studies to clinical medicine [55]. CDDP also generates ROS as well as reactive nitrogen species (RNS) in the cochlea, and several antioxidants have been shown to provide protection from CDDP-induced ototoxicity, including L-methionine, NAC, glutathione, and ebselen [56]. A potentially confounding factor is the propensity of some antioxidants to mitigate the efficacy of CDDP cancer treatments. An appropriate choice of the preventive agent or local cochlear application may circumvent this problem [57,58].

Prevention of acoustic trauma

The mechanisms of acoustic trauma-induced pathology may be more complex than those of drug-induced ototoxicity, as they include not only the formation of ROS but also calcium influx, vasoconstriction, and direct mechanical damage to the organ of Corti at higher intensities [59,60]. In addition, the sources of noise trauma can be highly variable, ranging from impulse noise in form of a single, high-energy burst, such as an explosion to chronic noise exposure at various levels of intensity. These conditions may induce different variations of stress or variations in the response of cell death and survival pathways. Furthermore, while a protective antiototoxic therapy can be timed with drug administration, patients may report noise-induced hearing loss (NIHL) days, months, or years into the trauma. To what extent a delayed intervention may be successful is yet another question that remains to be resolved.

There is good direct evidence that ROS are formed in the inner ear following intense sound exposures, specifically in the stria vascularis and the organ of Corti [61]. Indicators of ROS formation can be detected at the onset of the noise exposure and may persist for hours or even days following the exposure. Attesting to the importance of endogenous antioxidants, decreased glutathione in the inner ear enhances noise trauma, while dietary supplementation with glutathione attenuates these effects [62].

While the precise origin of ROS in the cochlea following noise exposure remains speculative, there are several sources most likely to contribute. A surge of superoxide radicals followed by chain-reaction formation of other free radicals (via Fenton-type reactions) may be due to the inefficiency of mitochondrial activity, prolonged tissue hypoxia due to vasoconstriction, and rebound hyperfusion [6,63]. RNS may additionally arise from the activation or induction of the enzyme nitric oxide synthase. The different isoforms of this enzyme serve a variety of physiological processes in normal tissue metabolism, and the product of the enzymatic reaction, nitric oxide, is an important second messenger molecule. When produced in excess, not only is nitric oxide potentially damaging as a free radical, but it can also combine with superoxide to produce the highly reactive and destructive peroxynitrite. Nitric oxide levels can rise after noise exposure [64,65], perhaps as a consequence of an enhanced release of excitatory amino acids [66,67], and may contribute to the overall pathophysiology of noise-induced hair cell loss.

Restoring redox balance is therefore one of the potential means of intervention in NIHL, another being an interference with cell death pathways. However, some approaches effective in animals may not be feasible for clinical application. For example, blocking cell death pathways succeeded in animal experiments in vivo [37,68], but similar concerns may arise here as in the prevention of drug-induced hearing loss, in that systemic intervention with important physiological processes may have unwanted consequences. Likewise, the upregulation of antioxidant defenses by systemically administered *R*-phenyl isopropyl adenosine has adverse physiological effects [69]. A local application may bypass systemic problem but is more invasive and may afford less control over drug concentrations. Another important point is that a protective treatment targeting a specific pathway may be counterproductive by inhibiting a potential rescue response. While reducing oxidative stress by scavenging ROS may be beneficial under certain stress conditions, early mild increases in ROS, such as under TTS-noise exposure, stimulate autophagy and remove cellular debris, allowing hair cell survival [40,70].

As with ototoxicity, antioxidant therapy is currently the most viable approach for the prevention and/or treatment of NIHL, with a variety of drugs as possible protective agents [59,60]. NIHL can be attenuated effectively by scavengers such as superoxide dismutase and allopurinol, antioxidants and iron chelators, and by antioxidant drugs that may have clinical appeal, such as ebselen or NAC [71]. Multidrug combinations are promising in animals, for example, acetyl-*l* carnitine with *d*-methionine or NAC [72,73]. Alternative strategies aimed at vasodilation and providing adequate blood flow to the cochlea during noise exposure may also indirectly attenuate ROS formation. Drugs such as pentoxifylline and sarthran improved blood flow to the cochlea and also reduced the temporary

effects of noise exposure [74,75]. Magnesium supplementation can maintain cochlear blood flow under noise exposure and reduce the resulting hearing loss [76]. A combination called "ACE-Mg" consisting of several antioxidants (vitamins A, C, and E) acting by different mechanisms and magnesium as a potential vasoactive agent has proven effective in preventing NIHL in animals [77,78]. While most strategies for interventions in animals rely on applying the protective treatment prior to or concomitant with noise exposure, evidence indicates that the formation of free radicals continues for days following noise exposure and that, consequently, an antioxidant treatment may still be effective during a short period post-exposure [65,79].

The translation from animal models to practical use in humans has been only partially successful in tests on populations exposed to high levels of noise during military exercises and leisure activities. These studies also clearly show the influence of genetic and physiological conditions on susceptibility to noise trauma and the limitations that these factors impose on protection. Daily supplementation with magnesium reduced the incidence of permanent threshold shifts (PTS) in hearing in military recruits from 21.5% to 11.2% [80] and, likewise, reduced temporary threshold shifts (TTS) in a group of young volunteers [81]. However, the severity of PTS was negatively correlated with an individual's monocyte Mg^{2+} content (which may be genetically regulated) regardless of treatment. As another example, NAC showed significant protection on TTS only in subjects with a null genotype in glutathione *S*-transferase T1 and M1 [82]. Perhaps not surprisingly, a group of nightclub visitors taking NAC sustained the same TTS as the control group [83].

Aside from the theory of ROS, calcium influx and overload after noise exposure may be the next most accepted notion. An involvement of calcium influx via calcium channels is supported by the fact that channel blockers attenuate NIHL. The L-type is the predominant VGCC in IHCs, but it may be also present in OHCs [84]. Under normal conditions, blocking of this channel reduces the calcium content of guinea pig IHCs and OHCs [85]. Delivery of L-type channel blocker diltiazem into guinea pig OHCs protects from acute noise damage [86], and several L-type channel blockers (diltiazem, verapamil, nicardipine, and nimodipine) attenuate noise-induced hair cell loss and auditory threshold shifts in female ddY mice [87]. In addition, T-type channel blockers also prevent noise-induced hair cell loss and hearing loss in C57BL/6 mice [88]. Calcium channel blockers are generally used to lower blood pressure in clinic so that their safety and potential side effects are known.

Multiple other cell death pathways, partly independent and partly overlapping, may be triggered by a spectrum of radicals generated in response to noise. The fact that both necrosis and apoptosis are observed attests to the existence of such multiple cell death pathways. This notion is also supported by the emergence of caspase-dependent and caspase-independent cell death pathways and the involvement of the transcription factors that code for different signaling cascades, such as AP-1, endonuclease G, and the calcineurin-related activation of BCL-2 family proteins [59,60]. The pan-caspase inhibitor ZVAD and receptor-interacting protein (RIP) 3 siRNA modulate noise-induced necrosis and apoptosis in OHCs by reduced caspases and RIP kinases, respectively. However, inhibition of noise-induced apoptosis shifts the prevalence of OHC death to necrosis adding an additional layer of complexity to pathway interactions and potential remedies [89].

Recently, epigenetic modification has shown promise to shed new light on strategies for prevention of NIHL as is the case for ototoxicity. FDA-approved HDAC inhibitor vorinostat (SAHA) attenuates noise-induced hair cell loss and hearing loss [41]. Furthermore, BIX 01294, a specific histone lysine methyltransferase inhibitor of G9a encoded by the human *EHMT2* gene, also reduces noise-induced hair cell loss and hearing loss [90] indicating suppression of genes by noise.

Antiinflammatory agents

There is increasing evidence that inflammation contributes to the loss of sensorineural elements from both ototoxic drugs and noise, and antiinflammatory agents are therefore another potential approach to prevention and treatment [91]. Reducing the inflammatory response by controlling signal transducer and activator of transcription (STAT)-dependent pathways decreased cisplatin ototoxicity [92]. Likewise, the corticosteroid dexamethasone protected against several ototoxic agents and against trauma from insertion of a prosthesis into the cochlea [93,94], as well as against noise exposure [95]. It should be kept in mind when considering prevention that the immune response can play an important and necessary role in response to stresses in the cochlea, and there may be occasions when enhancement rather than reduction provides benefit.

Heat shock proteins

HSPs constitute another natural protective mechanism against various forms of stress. HSPs can achieve their protective role by influencing the stress-related denaturation of proteins (either reducing the denaturation or enhancing renaturation and regaining the correct tertiary structure), as chaperones or through an influence on cell death cascades. Several families of HSPs (commonly designated by their molecular weights) include HSP 25/27,

HSP 32, and HSP 70/72. Expression of the inducible form of HSP 70 in the cochlea has been shown in response to heat [96,97], transient ischemia [98], noise [99], and ototoxic drugs [100].

A protective role for HSPs in the inner ear is suggested by two types of studies. In one type, HSP levels are upregulated by an initial innocuous stress, either low-level noise [101] (Altschuler et al., 2002) or controlled heat [96], followed by a noise exposure that would normally cause a significant hearing loss. Either of these preexposures resulted in a significant (30 dB) reduction in NIHL and hair cell loss. Similarly, preexposure to a heat stress protected hair cells in the utricle from aminoglycoside induced loss [102]. Geranylgeranylacetone, a drug that can cause induction of HSP 70 (HSP 70) provided protection from noise-induced [103] and CDDP-induced loss of hair cells [104]. Celastrol, another drug to upregulate HSPs attenuated aminoglycoside-induced hair cell loss in the utricle via HSP 32 induction [105]. HSPs can also protect hair cells from cisplatin (a cancer fighting drug)-induced cell death [106]. Transgenic mice overexpressing HSP 70 were less susceptible to kanamycin-induced hair cell loss and hearing loss [107]. In addition, increased HSP 70 alleviated hearing loss through the promotion of autophagosome formation and removal of noise-induced oxidative stress products [108]. The second type of study examined heat shock factor 1 (HSF1), a major transcription factor for the HSPs, present in hair cells and the stria vascularis in the rodent cochlea [109]. HSF1 knockout mice show increased hearing loss with increased loss of hair cells after noise exposure [110,111].

Neurotrophic factors

Neurotrophic factors play an important role in the development of the cochlea, in regulating differentiation of sensory and neuronal elements, and in the formation of afferent and efferent connections [112–114]. Many neurotrophic factors and/or their receptors remain expressed in the mature cochlea, including glial-line-derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3), and brain derived neurotrophic factor (BDNF) (Ylikoski et al., 1993) [114–117], where they play a role in cellular homeostasis. Neurotrophic factors have a protective action against stress through a mitigating influence on ROS or intervention in the cascade of downstream events induced by ROS formation. While removal of neurotrophic factors is proapoptotic, neurotrophic factors themselves can induce prosurvival pathways and provide protection. As a possible mechanism, neurotrophic factors may reduce oxidative stress–driven increases in intracellular Ca^{2+} [118–120] through the induced expression of calcium-binding proteins [121,122] or antioxidant enzymes [118,119].

Infusion of neurotrophins into the inner ear fluids prior to stress provides protection from hearing loss. When levels of GDNF or NT-3 are increased in the inner ear prior to noise overstimulation or the administration of ototoxic drugs, there is significant protection, with decreased hearing loss and hair cell loss [101,123–127, 134]. This protection is less effective if the neurotrophic factors are provided after the stress rather than before, suggesting that they play a greater role in protection than in repair. BDNF and fibroblast growth factor (FGF) were less effective in preventing hair cell loss [127,128].

Protection from excitotoxicity: "acquired" loss of auditory nerve connections to hair cells

The connection between IHCs and the AN is very sensitive to loss from the excitotoxicity generated by overrelease of the IHC transmitter, glutamate. This excitotoxicity can be induced by noise overstimulation or trauma to IHCs from hypoxia, changes in inner ear fluids or conditions that induce ROS in the hair cells. This can result in a "bursting" and loss of the unmyelinated portion of the peripheral process of the AN, normally connecting to the IHC. While there is potential for regrowth and reconnection [67] there is also potential for permanent loss. This can lead to subsequent death of the AN somata (spiral ganglion neurons—SGN) and loss of the central nervous system connection in the cochlear nucleus. A typical IHC may have connections to 10–30 AN peripheral processes (this varies depending on the position in the cochlear spiral and between species). Studies have shown that a mild to moderate level noise that produces only a temporary loss of hearing and no loss of sensory cells can still result in 20%–30% of these connections being lost, reducing the dynamic range of AN responsiveness [129,130]. Drugs that reduce excitotoxicity in the cochlea can be used to prevent or reduce the loss of IHC–AN synaptic connections. d'Aldin et al. [131] found that Piribedil, a dopamine agonist, reduced loss from a glutamate agonist. We found that the combinations of antiexcitotoxicity agents—Memantine, Piribedil, and ACEMg—when given prior to noise significantly reduced noise-induced loss of IHC-AN synaptic connections [132,133].

When there is loss of IHCs, a series of pathophysiological changes follow, including scar formation, a loss of the peripheral processes of the AN, and, over time, a substantial loss of the AN itself. This loss of AN can be related to the loss of survival/maintenance factors from hair cells and/or supporting cells. Loss of these survival/maintenance factors causes these auditory neurons to enter into the cell death cycle. Cochlear prostheses (discussed in a later section) depend on the stimulation of the

AN, so large AN loss would impair function. Replacing lost survival factors can help maintain this population. Placement of GDNF, BDNF, or NT-3 into cochlear fluids following IHC loss significantly reduced subsequent loss of SGN [115,123,134–138]. Chronic electrical stimulation also reduced SGN loss [139–144]. The combination of neurotrophic factors and ES is particularly effective [145,146].

Infusion of neurotrophic factors such as BDNF, NT-3, and FGF can induce regrowth of the peripheral process of the AN that regress following IHC loss [101,138,147,148]. There is interest in inducing the regrowth of AN processes toward specific sites on the cochlear prostheses. Cochlear prostheses might have reduced thresholds if their target is closer, making them more energy efficient and reducing battery requirements, and it might also allow a greater number of channels to be used, resulting in more and better frequency separation.

Neurotrophic factors delivered into cochlear fluids can also be used to induce IHC-AN reconnection when hair cells are not lost. NT-3 delivered into cochlear fluids by poloxamer on the round window, given 1 day after a noise, induced significant reconnection of IHC-AN synaptic connections [149].

Gene transfer for the prevention and treatment of genetic deafness

One in 1000 newborns suffers from hearing impairment. In developed countries, more than half of the afflicted babies are deaf for genetic reasons. Genetic inner ear impairments can be nonsyndromic, affecting only hearing, or syndromic, with multiple defects occurring in several body systems. Many of the genes for deafness, as well as their products, have now been identified, with mutation of connexins being the most common cause of nonsyndromic deafness in people. Many stereocilia-related mutations have also been found, including in several myosins and adhesion proteins [150]. Identification of the genes that are mutated in families with genetic inner ear impairments is important not only for diagnostic purposes but also for the potential for prevention and cure. Gene transfer has been successful in mouse models if applied early enough. Probst et al. [151] demonstrated that in transgenic "shaker-2" mice destined (genetically) to become deaf, insertion of the wild-type (correct) myosin MYO15 gene sequence into the fertilized egg corrected the genetic deficit, leading to normal inner ear structure and function in the adult mouse. This demonstrated that addition of the correct copy of the gene can rescue the inner ear from genetic deficits. Mice lacking vesicular glutamate 3 (VGLUT3) are born deaf and adeno-associated virus (AAV)–mediated gene transfer of VGLUT3 into IHCs partially restored normal cochlear morphology and hearing [152].

Development of gene transfer into the cochlea has therefore been a major focus of research. One application has been to induce repair after deafness (discussed later) and the other application has been to prevent or treat genetic deafness. There have been many successes in mouse models of human deafness. Several groups have shown full or partial restoration of hearing with neonatal gene transfer into cochlear hair cells in mouse models of Usher syndrome (e.g., [153–156]). György et al. [154] were also able to show gene transfer in the cochlea of nonhuman primate. Chang et al. [157] used neonatal AAV gene transfer to restore hearing in a mouse model of Jervell and Lange-Nielsen deafness syndrome. Chien et al. [158] rescued hair cell morphology in deaf whirler mice.

There are many remaining challenges in bring the use of AAV related vectors to human application for cochlear gene transfer; these include the following: (1) gene transfer in mice is most successful in early cochlear developmental periods—corresponding to in utero in human, when human application would be more difficult. (2) The cochlear cell types where there is best gene transfer may not always correspond to the needed treatment target(s), (3) there can be limited transport cargo capacity in many AAV vectors that can limit gene transfer; (4) potential for immune response and damage from the vector must be limited. A recent study addressed two of these challenges, using a dual AAV approach, each with a different recombinant vector, to correct mutation in the otoferlin gene that caused DRNB9 a common genetic deafness [159]. This was also successful at a later age in mice, which could correspond to neonatal in human.

Interventions for hair cell repair: gene therapy for transdifferentiation

While cochlear type hair cells in nonmammalian vertebrates are naturally capable of regeneration [160,161], in mammals there is no such regeneration, and once mammalian hair cells are lost, there is no natural replacement by new hair cells [162]. The main approaches for replacing hair cells are the introduction of new cells from an exogenous source such as stem cells, or converting endogenous cells that have a nonsensory phenotype to become new hair cells [147,163–166]. Since there is little evidence of endogenous stem cells in the mammalian cochlea, other endogenous cells would need to dedifferentiate and then redifferentiate into hair cell; this is called transdifferentiation, one of the mechanisms utilized for hair cell regeneration in chick and nonmammalian vertebrates [167–170]. Naturally occurring transdifferentiation of less mature supporting cells can occur during the periods of cochlear development in mammals, early postnatal

periods in mouse and rat models (e.g., [171]). This ability of mammalian supporting cells to transdifferentiate, however, disappears as the cochlea further matures, suggesting epigenetic regulation that turns off this capacity ([172] for review). There has therefore been considerable recent research on genes and signaling pathways that regulate the proliferation and differentiation of hair cells during cochlear development ([173,174] for review; [175–181]). As mentioned in the previous section, there has also been considerable research on development of gene transfer and gene therapy, and this has been used both to test hypotheses on the signaling pathways and to test for inducing transdifferentiation and repair/replacement.

The function of several gene families in hair cell development has been characterized by manipulating expression of these genes in mice. In general, these transgenic manipulations use several strategies for increasing or decreasing the levels of gene expression, in fashions that can be temporally regulated and cell specific. Cre-loxP and CRISPR technology has been especially useful for inducing conditional gene expression inhibition in a cell-specific manner in the inner ear [182]. With the use of mouse transgenics, it is possible to eliminate the generation of hair cells [177,183] or to induce ectopic hair cell formation next to the organ of Corti [184,185].

Hair cells and supporting cells develop from common progenitor cells [175]. Their phenotype is determined by a set of genes expressed in a sequence and influencing neighboring cells in an interactive way, with additional influence of microRNAs [186,187] and other epigenetic regulators [172]. *Atoh1* is a gene encoding a transcription factor that is necessary and sufficient for hair cell differentiation in the developing epithelial ridges [177,183,188]. Since *Atoh1* is a master regulator gene for hair cell development, manipulating its expression levels has been used to generate new hair cells both in transgenic animals and by overexpression using other methods. Ectopic hair cells could be found after virally mediated expression of *Atoh1* in nonsensory areas of the auditory epithelium in a mature guinea pig ear in vivo [189,190] and in culture [191]. Together, these studies have shown that the nonsensory cells retain the competence to respond to developmental genes and to convert to new hair cells after the onset of deafness, but the ability of the auditory epithelium to respond to overexpression of Atoh1 appears to diminish in mature animals and after the tissue is severely traumatized [192,193]. New hair cells induced by the overexpression of *Atoh1* had the surface morphology of hair cells, but their detailed morphology is immature [190,194]. Nevertheless, new hair cells that are generated by *Atoh1* overexpression are electrophysiologically active, and several aspects of the maturation process recapitulate the normal development of hair cells ([192,195].

Gene therapy is used to manipulate levels of specific proteins in cells and tissues. It can also be used to inactivate specific proteins or to overexpress them. In most cases, this is accomplished either by introducing a foreign gene into the target cells or by using siRNA approaches. Viral vectors are the most efficient vehicles for gene transfer. Adenovirus, herpes simplex virus, AAV, and lentivirus can mediate gene transfer into cells of the inner ear [165,196–201]. Gene-transfer protocols can be used either for influencing the target cell proper or to accomplish secretion of diffusible factors that act in a paracrine way. Example for the former is the overexpression of Atoh1 for inducing transdifferentiation of supporting cells to new hair cells. Examples for therapy with genes encoding secreted and diffusible proteins include neurotrophin therapy, used for the protection of epithelial and neural elements in the cochlea [44,125,136,202,203].

Because hair cell loss depletes the cell population in the organ of Corti, some of the genetic manipulations performed on mice were aimed at influencing proliferation in the cochlea. This is important because it may be possible for some of the new cells to become new hair cells without any further exogenous manipulations. In the normal mature ear the $p27^{Kip1}$ gene is expressed in nonsensory cells and keeps them quiescent [204,205]. Mice with deficient expression of the $p27^{Kip1}$ gene exhibit an excessive number of hair cells in the organ of Corti. It appears that in the absence of cell cycle arrest, cells continue to proliferate after they normally would have stopped, had $p27^{Kip1}$ been active. Interestingly, mice with this induced mutation have a severe hearing loss, despite the large number of hair cells [205,206]. It is currently unclear whether the hearing deficiencies are due to dysfunction of the hair cells proper or due to more general cochlear pathologies related to the presence of ectopic hair cells. Inhibition of the cell cycle can also be removed by blocking the expression of other genes, including Ink4d and Rb1 [207–209], leading to supernumerary hair cells and supporting cells in the tissue. The ability to use genetic manipulation for inducing proliferation in the organ of Corti is a considerable breakthrough that can be combined with transdifferentiation therapy and lead to feasible clinical applications.

There are several challenges remaining for gene therapy to induce regeneration or treat deafness-related pathology; these include (1) reaching the appropriate target population/cell type in the cochlea; (2) the target population of cells (auditory epithelium) changing as scar-formation progresses following hair cell loss; (3) the ability of vectors to carry sufficient load of genetic material to transfer; and (4) the vector not inducing immune response or damage.

The challenge of reaching the appropriate target cells has been partly met by placement of vectors into the scala

media that induces expression of transgenes in the supporting cells in animal models [210–212]. Still, this route of inoculation is not easily accomplished in the human ear.

Interventions for repair: hair cell and auditory nerve replacement—exogenous stem cells

The use of exogenous stem cells is another approach for hair cell replacement as well as for replacement of lost AN. Embryonic stem cells or stem cells derived from bone marrow can be induced to reach a hair cell-like phenotype (e.g., [213–221]). There has now been successful generation of organoids containing cells with hair cell phenotype [222–224]. Two major challenges for hair cell replacement however remain, efficiently generating large numbers of stem cells reaching the needed hair cell-like phenotype as well as placing these into an appropriate niche in the cochlea to allow function.

Embryonic stem have been induced into an AN-like phenotype both in vitro and in vivo ([225] for review). The in vivo studies have shown that mouse embryonic stem cells can be placed into the cochlear fluids of scala tympani or directly into the AN, survive, differentiate into a neuronal phenotype [226–231] and can migrate into the remaining spiral ganglion cell population [232]. Stem cells that reach a neuronal phenotype will send processes that reinnervate hair cells [233,234] and have been shown to make connections in the cochlear nucleus and improve auditory brain stem responses (e.g., [8,9]). Differentiation into an AN-like phenotype typical of SGN can be improved by genetically engineering the stem cells to produce factors that guide AN differentiation during normal development and/or by providing neurotrophic factors that influence the development of the SGN phenotype [227]. Challenges remain for making appropriate central nervous system connections.

Interventions for repair/replacement: cochlear prostheses

Auditory prostheses can provide direct stimulation of the auditory pathways for return of hearing following deafness. In severe and profound sensorineural hearing loss the sensory cells of the cochlea are destroyed. Cochlear prostheses bypass the damaged receptor epithelium and electrically excite the AN fibers directly. The development of the cochlear prosthesis has been the success story of the field of neuroprotheses. It has provided a therapy for the profoundly deaf, where none previously existed. The first FDA-approved inner ear implants, approximately 30 years ago, were single-channel devices that provided crude input and were an aid to lip reading. Now multichannel devices, combined with many advances in signal-processing strategies, routinely provide intelligible speech perception with open set speech discrimination and even use of the telephone in the majority of implant recipients (e.g., [235,236]). With the success of cochlear implant performance and the low risk associated with its placement, the age at which implantation is considered has decreased to where very young children receive implants and show benefit from early placements, taking advantage of critical periods for auditory system development. There is also increasing bilateral placement to allow binaural hearing, important for spatial localization [236–238]. Recently, the candidate pool for cochlear implants has expanded to include people with remaining hearing. Shorter implants provide electrical stimulation in the basal cochlea and acoustic stimulation remains possible in the more apical cochlea. The use of such "hybrid electrodes" allows acoustic—electrical hearing with improved speech recognition, particularly in noise and subject also report better appreciation of sounds such as musical melodies [239].

Fully implantable cochlear prostheses

The typical currently used cochlear prostheses/implants (CI) have an external microphone/processing unit, a radio frequency inductive link, and intracochlear electrodes. Although the CIs enable speech recognition, they have major limitations including high cost, high power consumption (20–40 mW), cosmetic concerns, and safety issues associated with the external processing unit [240–242]. These limitations contribute to an international market penetration for CIs of approximately 0.7% [243]. A fully implantable cochlear implant is attractive, because it would improve cosmetic and safety issues. Unlike traditional CI where external components must be removed for many activities including sleep and showering [244], a fully implantable CI could remain on at all times. One key advantage of a fully implantable cochlear implant would eliminate the inductive link between the processor and CI electrodes that causes 60% of the power drop [241] in the device, thus reducing power consumption [240].

There have been several attempts to build fully implantable cochlear implants [245–250]. Each requires an internal microphone of some kind. For instance, Cochlear Corporation developed the totally implantable cochlear implant (TIKI), which used a subcutaneous microphone. In a clinical study of this device [246], subjects reported benefits from TIKI and had continued to use it on a daily basis because of cosmetic advantages and the ability to hear while showering, sleeping, and doing physical work. However, speech

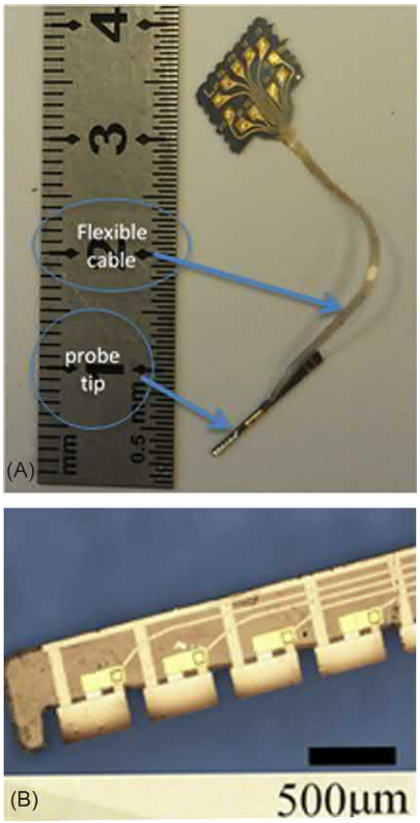

FIGURE 59.1 Prototype of intracochlear pressure sensor [255]: (A) shows prototype including five-element probe tip (elements are submillimeter and too small to be seen in 4A, see 4B for micrograph). Only the probe tip is inserted into cochlea. This configuration includes a flexible parylene gold cabling that terminates in an electrode bay, which enables functional testing of the cochlea exterior to the cochlea. (B) Probe tip close-up showing piezoelectric bimorphs.

perception results were significantly lower when compared with the traditional CI because of the reduced sensitivity and increased body noise contamination of the subcutaneous microphone. The TIKI has not been adopted in great numbers, however. The state-of-the-art for implantable acoustic sensors for hearing devices is well reviewed (e.g., [251]) where they also present a transtympanic microphone to measure ear canal pressure for hearing aids or cochlear implants. This approach, while promising, also faces issues such as ventilation tube migration and liquid contamination which would reduce the effectiveness of the device [251]. An intracochlear transducer is an attractive alternative for patients with a functioning middle ear, because the intracochlear acoustic pressure is typically higher than the ear canal pressure. Implantable acoustic sensors must be small. Creighton et al. [252] and Pfiffner et al. [253] implanted a miniature microphone in the cadaveric human temporal bone and measured intracochlear sound pressure in vitro. Recently, a PVDF-based intracochlear microphone prototype was developed and used to measure sound in a living gerbil cochlea [254]. Zhao et al. [255] (Fig. 59.1) have a different approach to fabricating an implantable acoustic transducer, one based on an aluminum nitride (AlN) microelectromechanical systems xylophone along with physiological testing in a living guinea pig to sense the voltage response due to external acoustic excitation.

This sort of micro-piezoelectric technology includes its use in an intracochlear speaker (receiver) for a hearing aid or in a hybrid electroacoustic stimulator for a cochlear implant. In current prosthetics the stimulation is extracochlear, either outside the tympanic membrane or in the middle ear [256]. Luo et al. [257,258] fabricated and used just such an intracochlear PZT (lead zirconante titantate) actuator to evoke an auditory brainstem response by generating acoustic signals inside a cochlea. Although there are some questions regarding the biocompatibility of a produce containing lead, it might be possible to shield the patient from lead, and certainly other lead-free materials are available (e.g., AlN or PVDF) [255].

Another approach to enabling a completely implantable CI is to replace the external digital signal processor for a cochlear implant with a cochlear analog processor. The goal of such an approach is to miniaturize the processor and reduce the power required to do the computations compared to the traditional digital processor. In addition, an analog processor holds the potential to do the nonlinear computations in real time. Two approaches to such an analog computer are (1) a microelectromechanical system analog model (e.g., [259]) or (2) an analog integrated circuit model (e.g., [260]). While a fully implantable CI will provide for a more usable device, miniaturization of the acoustic sensor and interface to the electric neural stimulator are needed, and the device will still need power and energy storage. All these aspects must be part of the larger engineering design to make the fully implantable device a reality.

Interventions for repair/replacement: central auditory prostheses

Central auditory prostheses can be advantageous when the cochlea is not suitable for implantation, when there is insufficient AN survival, or when AN must be removed in patients with VIII nerve tumors (acoustic neuroma). While a peripheral cochlear implant allows for more "normal" auditory pathway processing, the central implants can take advantage of the tonotopic organization of auditory nuclei for frequency separation. Central auditory prostheses can also have reduced thresholds because of the close proximity to neurons as well and increased dynamic range. Cochlear nucleus implants have been successfully applied in many subjects, with excellent performance possible

[261,262] in nontumor patients; however, when there is a tumor (the most frequent need for central auditory prostheses), the complications of the tumor and its removal can compromise the long-term function of the cochlear nucleus implant (e.g., [263]). Auditory prostheses have also been applied in the inferior colliculus in both humans and in animal models [264] with animal models showing improved thresholds, increased dynamic range, and better frequency discrimination than cochlear implants [265].

Local delivery to cochlear fluids

The fluid spaces of the cochlea provide for a closed environment well suited to receive local delivery of chemicals. Local delivery provides improved access and avoids the side effects that systemic delivery could entail. One method of local delivery utilized in animal models is miniosmotic pumps with cannula into the inner ear fluid of scala tympani or into the middle ear with access through the round window has been developed in animal models (e.g., [266]) and effectively utilized for local application of chemicals that provide protection from acquired deafness or enhanced survival of AN (see earlier sections). While devices for fluid delivery to the inner ear can be developed for human application [267–269], many recent efforts have focused on combining fluid delivery with cochlear prostheses since combining electrical stimulation and chemical delivery is more effective in enhancing AN survival following deafness than either applied by itself [145,146]. Moreover, more patients with some residual hearing have been shown to benefit from cochlear implants and more patients with surviving hair cells are now considered candidates for cochlear implants. These subjects would benefit from the protection of these remaining hair cells from the trauma of cochlear prosthesis insertion. There is also the potential of inducing regrowth of peripheral processes toward the stimulation sites, which could lower thresholds and enhance selectivity and separation. Therefore recent efforts have been made by cochlear implant manufacturers and research groups to develop cochlear prostheses capable of both electrical stimulation of the AN and delivery of pharmaceuticals into the cochlear fluids [270]. These have been successfully applied in animal studies [146] and clinical application is beginning.

Biopolymers and nanoparticles can be used for timed release of specific factors. Nanoparticles can be placed directly into the cochlear fluids (e.g., [271–274]). These can be designed with different sizes, shapes, and release properties and can also be designed to target specific cochlear elements. They can also be placed directly on prostheses. Biomaterial can also be placed on prostheses that can not only improve function and histocompatability but also provide a means for delivering therapeutics. Specific placement of biopolymer and microchannels could provide different factors to different regions.

Another approach to delivery from prosthesis is to place cells capable of secreting the substance of interest directly onto the prosthesis, where they can deliver to cochlear fluids after placement into scala tympani. With ex vivo gene transfer, cultured cells are transformed to produce a specific gene product and placed into a specific area of the body for region-specific release. One problem has been that the transformed cells can migrate to a different region. With the use of biopolymers with fibronectin or laminin, transformed cells (e.g., fibroblast and fibronectin; Schwann cells and laminin) can be securely attached by the biopolymer to a neuroprobe or prosthesis, which is then inserted into the region of interest. The cells will then remain in place and have their highly localized action. One such study has recently shown the ability of BDNF, secreted by cells attached to the implant, to enhance the survival of SGN in a deafened guinea pig ear [275]. It may also be possible to place stem cells (discussed previously) on a prostheses, either to release drugs of interest or as new neurons close to the stimulation sites that can create a new connection either directly to the central auditory system or to remaining SGN.

The middle ear more easily accessible than the inner ear fluids and drugs can be delivered into the middle ear through the tympanic membrane (ear drum). This can be for middle ear infections but can also cross the round window and diffuse into the cochlear fluids. Slow release formulations have been developed that can be placed close to round window for this purpose [276]. These have not only been used in preclinical animal studies for dexamethasone [277], ciprofloxacin [278], and testing NT-3 delivery [149] but have now moved into clinical application for treating Meniere's disease.

Conclusion

Major advances in our understanding of the molecular mechanisms underlying deafness and of the factors that influence and modulate its expression and progression have occurred since the mid-1990s. We can expect, as progress in these areas continues, that this knowledge will form the basis for *molecular otology*. Novel tissue engineering–based therapeutic interventions may become a major part of the practice of otolaryngology in the 21st century.

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Part Sixteen

Ophthalmic

Chapter 60

Stem cells in the eye

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Introduction

A stem cell is defined by its capacity for differentiation and self-renewal. Stem cells are generally divided into two broad categories, embryonic stem cells (ESCs) and adult stem cells. The ESCs are derived from embryos at a developmental stage and are pluripotent, that is, a cell that has the ability to differentiate into all the types of cells that are in all three germ layers. During replication, ESCs retain their undifferentiated state; after appropriate stimulation, ESCs can differentiate into lineage-specific cells. However, ethical controversies exist in the use of ESCs for scientific research. Therefore adult stem cells have been also explored in research and tested in clinical studies. In contrast to ESCs, adult stem cells are multipotent, and thus they can only differentiate into cells of a given germ layer. Pluripotent stem cells (PSCs) are similar to ESC and have the ability to differentiate into cells of mesoderm, endoderm, and ectoderm lineages. PSC can be reverted from adult cells through two different mechanisms: somatic cell nuclear transfer and by induction with transcription factors into inducible PSC (iPSC). Both approaches have the potential to generate "patient"- specific PSCs.

The eye is a complex organ consisting of epithelial, mesenchymal, connective, and neural tissue. Vision is dependent on carefully regulated structural and functional integration of these tissues. Over the last two decades, it has become increasingly apparent that there are a number of stem cell niches in ocular tissues that are important in maintenance and repair. Moreover, there are nonocular-derived stem cells from sources exogenous to the eye that has been developed as therapies for retinal degeneration diseases (Fig. 60.1). *This chapter will consider our current knowledge of ocular and nonocular stem cells and examine their therapeutic potential in repairing damage to ocular tissue.*

Endogenous ocular stem cells

Corneal stem cells

Epithelial stem cells

The corneal epithelium is the outermost corneal layer. Its functions include transparency and protection from external environment. Corneal epithelium comprises 5−7 layers of cells and is a stratified squamous epithelium that constantly sheds the uppermost dead cells and is replenished from the peripheral stem cell source during homeostasis. The fairly rapid cell turnover contributes to a uniform structure and corneal thickness avoiding transparency loss. Corneal epithelium is renewed and repaired throughout life by the limbal epithelial stem cell (LESC) pool residing at the peripheral corneoscleral junction called the limbus that separates cornea and conjunctiva [1,2]. The distribution of LESCs does not appear to be uniform; the population of LESCs shows regional differences, with the greatest number being in the superior and inferior quadrants [3,4]. LESCs are slow-cycling and normally quiescent cells and are part of the basal layer of the multilayered limbal epithelium. During wound healing, they become activated and their progeny ensures epithelial wound closure [5]. In humans, LESCs are located in a specific limbal niche called palisades of Vogt and may also be found in the deeper epithelial crypts [3,6,7]. Their progeny called transient amplifying cells (TACs) migrate toward the central cornea and upward from the basal epithelial layer. LESC location at the limbus and centripetal migration of their progeny has been recently confirmed in several studies using Confetti mice with color-based lineage tracing of individual epithelial stem cells [8−10]. Central corneal cells further divide and differentiate before being shed from the epithelial surface as terminally differentiated superficial cells.

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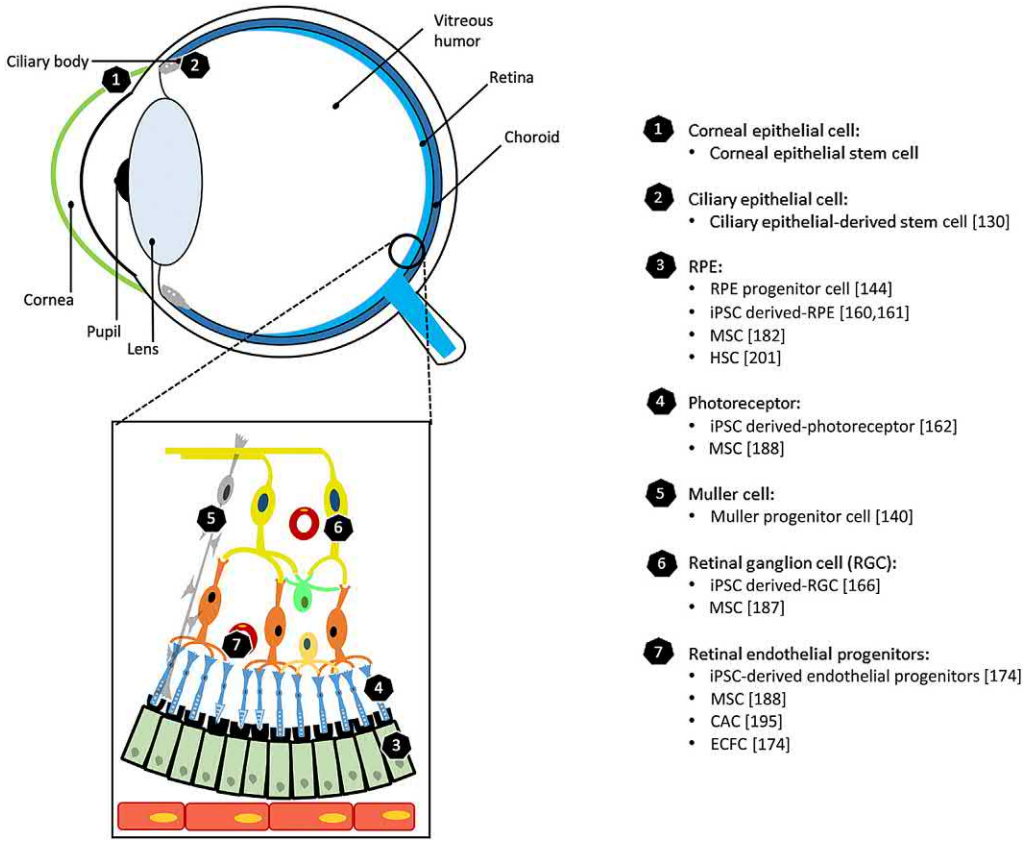

FIGURE 60.1 Ocular stem cells are important in maintenance and repair ocular tissue. Nonocular stem cells are developed as therapies for retinal degeneration diseases.

Each epithelial stem cell maybe capable of producing TACs throughout one's lifetime, but a fraction of LESCs could remain in the resting G_0 state for at least part of their lifetime [11–13]. LESCs have been characterized based on their colony-forming ability (forming holoclones), proliferative potential, slow-cycling nature (BrdU or EdU label-retaining cells), expression of specific markers, and lack of terminal differentiation markers [13–15]. A number of markers [16] sequestered to the limbal epithelium were proposed for identification of LESC but all of them eventually were ruled out as specific stem cell markers. For this reason, investigators have used a set of putative LESC markers (Table 60.1) to assess the fraction of progenitor cells in cultures and predict transplantation outcomes in pathological conditions [9,18,19].

The limbal stem cell niche

Epithelial stem and progenitor cells in the limbus intimately interact with stromal cells in a highly vascularized environment. The general contention is that epithelial-adjacent stromal mesenchymal components forming the limbal stem cell (LSC) niche are important for maintenance and support of LESC [3,20]. These vimentin-positive cells have a capacity to support limbal epithelial cell expansion in vitro and are closely associated with epithelial cells, so that they are coisolated upon collagenase digestion [21]. The precise molecular mechanism by which the stromal niche cells regulate LSC fate is unclear, although it is likely to be due to intricate and direct interactions between the stem cell and its microenvironment [22–24], as well as short- and long-range signals. Recently, a role of exosomes in maintaining epithelial-stromal communications has been suggested [25,26].

Regulation of limbal epithelial stem cells and transient amplifying cells

The regulation of stem cell homeostasis involves both internal and external factors. The environments associated with LESCs and TACs differ in several key ways and are critical to determining cell fate in each region [27,28]. Notably, there are differences that exist between the extracellular matrix/epithelial basement membrane that underlies these cells in the limbus and central cornea, including distinct expression of laminin and collagen IV

TABLE 60.1 Positive and negative markers of limbal basal epithelial cells [16,17].

Negative markers of LESCs	Limbal basal cell markers
K3/K12	ABCG2/BCRP1
Connexin 43	K5/K14
Connexin 50	K19
P-cadherin	Vimentin
E-cadherin	ΔNp63α
Involucrin	ABCG2
Integrin α2	Integrin α9
Integrin α6	K15
Integrin β1	K17
Integrin β4	Integrin β5
Nestin	ABCB5
NGF-R TrkA	C/EBPδ
Desmoglein-3	Bmi1
α-Enolase	Notch-1
	Frizzled 7
	SOX9
	TCF-4
	Periostin
	Wnt7a
	Actinin 1

LESC, Limbal epithelial stem cell.

isoforms, tenascin-C, collagen XII, etc. [29–31]. Other important effectors include cell adhesion molecules, cytokines, and growth factors that modulate corneal wound healing responses through complex mesenchymal-epithelial interactions [5,32] and are thought to play important roles in the regulation of LESCs and TACs. Depending on the interactions that cytokines are involved in, they can be assigned to groups. Epithelial cells secrete Type I cytokines [transforming growth factor-β (TGF-β), interleukin 1β (IL1β), platelet-derived growth factor-BB (PDGF-BB)] to modulate fibroblasts. Mediators of both epithelial and fibroblast cells are Type II cytokines [insulin-like growth factor 1 (IGF1), TGF-β1, TGF-β2, TGF-β3, FGF-2]. Corneal epithelial cells produce Type III cytokines [keratinocyte growth factor (KGF/FGF-7), hepatocyte growth factor, various epidermal growth factors (VEGFs), and opioid growth factor/5-met-enkephalin]. The interplay between epithelial cells and stromal fibroblasts in the central cornea and limbus is likely to influence cell behavior and phenotype [33–37]. A good example is the presence of TGF-β1, 2, and 3 and receptors in the limbal epithelium. TGF-β inhibits LSC proliferation [35] and may therefore influence stem cell maintenance in the limbus. Stem cell and TAC are regulated by many cytokines/growth factors, including the Notch and Wnt systems [38–43].

Evidence of corneal epithelial cell plasticity

Possible plasticity of corneal epithelial progenitor cells has been suggested by recent data. In the limbal area of mouse eyes, clusters of cells (compound niches) expressing goblet cell mucin and keratins 8 (conjunctival) and 14 have been found and increase in number during wound healing [44]. Another example came from Di Girolamo's group that transplanted keratin 14-positive mouse corneal epithelial progenitor cells onto corneas with experimentally induced to LSC deficiency (LSCD). After transplantation, these cells were able to transdifferentiate into keratin 8-positive conjunctival and keratin 10-positive cutaneous epithelia [45]. Interestingly, ablation of LESC can trigger the expression of progenitor marker keratin 15 in central corneal cells that dedifferentiate to progenitor cells during wound healing [46]. These findings suggest that corneal epithelial progenitor and even differentiated cells can be reprogrammed. The mechanisms that control these processes are still to be determined.

The pursuit of corneal epithelial stem cell markers

LESCs are operationally recognized as slow-cycling label-retaining (BrdU or EdU) cells residing in the basal cell layer of the limbus [13]. This small population (about 2%–3%) of cells has a high nuclear/cytoplasmic ratio [47,48] and is relatively undifferentiated as demonstrated by their lack of epithelial differentiation markers, such as 64 kDa keratin 3, cornea-specific keratin 12, and connexin-43 [16,17,49–58]. LESC progeny (TACs) are rapidly cycling cells that are thought to form the bulk of limbal basal epithelium and coexpress vimentin and keratin 19 [52]. As TACs migrate centripetally across the limbo-corneal margin and in the process acquire differentiation markers, for example, keratins 3 and 12 [28,49,56]. At least in some species, they might retain some progenitor markers as well, which would explain data on the expression of such markers also in the basal cells of the central mouse cornea, although this does not happen in humans [54].

To date, no definitive and specific marker characterizes LESC. Currently, negative LESC markers as well as some putative positive markers have been proposed [5,9,16,17,19,48,58]. Table 60.1 shows a list of the available negative and positive markers that maybe used to

discriminate basal limbal epithelial cells from basal central corneal epithelial cells. When used in combination, these markers can aid in LSC isolation and characterization of their differentiation state. Most putative stem cell markers are not exclusively expressed in a subpopulation of cells in the basal limbal cell layer, but they are rather found across the limbal basal epithelium and only a selected few (e.g., N-cadherin) show clustering in this layer compatible with LESC. High expression of the transcription factor ΔNp63 was proposed as a potential LESC marker for predicting success of expanded limbal cell transplantation [51]. However, the limbal basal epithelial cells, including LESC, express predominantly one p63 isoform (ΔNp63α) but the available antibodies do not specifically recognize it creating some confusion when interpreting data.

In the limbus, estimations for the stem cell population that maintains the corneal epithelium indicate less than 5% of the basal limbal epithelial cell population [17,47,48] or 100 cells in the rodent cornea. Like other stem cells, a subpopulation (side population on flow cytometry) of limbal epithelial cells, approximately 0.4%, is able to efflux Hoescht 33342, a property attributable to the ATP-binding cassette transporter G2, or ABCG2 [51,57–59]. A study of the rabbit limbal epithelial side population cells showed that they represented about 0.7% of the total population, were small in size, were undifferentiated and noncycling, and could be induced to enter the cell cycle upon wounding [51]. Whether the side population cells proliferate and form colonies in vitro remains controversial [51,59].

Overall, researchers still need to use several putative LESC markers together. The definitive identification of LESC markers may soon become reality with the advent of new technologies, such as time-controlled GFP expression [17] and single-cell RNA sequencing.

The potential for tissue engineering of limbal epithelial stem cells in ocular surface disease

Loss or dysfunction of LESC leads to LSCD that may result from mechanical injuries, chemical and thermal burns, genetic defects (e.g., aniridia, Stevens–Johnson syndrome), or chronic diseases, leading in severe cases to conjunctival ingrowth with neovascularization, corneal opacity, scarring, ulceration, and in some instances, corneal perforation and eventual vision loss [5,56]. Central corneal button without inclusion of the limbal region will not restore vision. Initially, autologous conjunctival transplantation was attempted to treat LSCD-related ocular surface disease [60–62]. However, in the absence of the limbal epithelium, conjunctivalization of the cornea resulted in an abnormal corneal surface [63,64], because "conjunctival transdifferentiation" does not occur [64–66]. Then, other methods were investigated, including keratoepithelioplasty, consisting of the transplant of lenticules of peripheral cornea [67] and human keratolimbal autografts were performed in the late 1980s [68]. A recent variant of such grafts for unilateral LSCD called simple limbal epithelial transplantation consists of placing small pieces of an autologous limbal biopsy on human amniotic membrane glued or sutured to a diseased eye. Cells migrate out of these pieces and substitute missing LSCs [69,70]. Human limbal autografts use the limbal cells of the healthy contralateral eye, whereas bilateral disorders necessitate stem cell allografts isolated from living tissue-matched eyes or nonmatched cadaver eyes [63,71,72]. In the case of allografts, immunosuppression is required to prevent epithelial rejection.

It should be noted that limbal biopsies from a healthy eye are not entirely safe, because stem cells do not repopulate the biopsied area. For this reason, and for bilateral LSCD treatment, the culture-expanded LESC therapy has been developed. This concept minimized the risk of LSCD in the donor eye [73], since only a small biopsy was required, and allograft rejection was reduced due to the elimination of Langerhans cells during culture [74]. Pellegrini et al. [72] reported the first successful autologous graft for unilateral severe ocular surface disease, using limbal epithelial cultures enriched in stem cells. Since then, culture-expanded LESCs have been used with reasonable success for transplantation in India, the United Kingdom, and Germany [75], and the first standardized cultured LESC treatment was approved in the European Union in 2015 [16,19].

A number of substrates for the culture of epithelial stem cells have been introduced, including fibrin, amniotic membrane, and thermo-responsive plastic [19]. The latter allows cultured epithelial cells to be released upon temperature change, so that only the epithelial sheets are transplanted [76]. The fibrin gel used as a carrier [72] is degraded following transplant, whereas the amniotic membrane appears to persist following transplantation and is only gradually resorbed [77]. Amniotic membrane remains the most used substrate for cultured LESC transplantation. Its benefits include provision of growth factors important in reepithelialization, antiinflammatory effects, inhibition of conjunctival fibrosis [78], and antibacterial properties. It is also thought that the amniotic membrane provides LESCs with a new niche, especially that the amniotic basement membrane is similar to the limbal epithelial basement membrane in composition [79,80]. A number of studies have evaluated the use of intact versus denuded (with epithelial cells removed mechanically or enzymatically) amniotic membrane as a substrate for LSC culture [80–83]. Limbal epithelial cells demonstrated stem-like properties on intact amniotic membrane, including slow-cycling and lack of expression of the

differentiation markers keratins 3 and 12, and connexin 43. However, cultivation on denuded amniotic membrane showed increased limbal epithelial cell migration and produced a better stratified epithelium [81].

Bioengineered epithelial tissue equivalents have been derived from both limbal explants and cell suspensions isolated from limbal epithelium [81]. Both techniques generate epithelial cell sheets that express differentiated markers keratins 3 and 12, although they are more evident in superficial cells of the cell suspension−derived sheets. The latter also resulted in the formation of a greater number of desmosomes, smaller basal intercellular spaces, and secure attachment via hemidesmosomes to the underlying basement membrane. The use of the air−liquid interface method of culture, whereby the level of media is reduced below the epithelial cell surface, ensures that a well-differentiated, stratified epithelial tissue is produced for transplantation.

Donor epithelial cells have been identified in the recipient bed for up to 30 months after limbal allograft transplantation [84], but the long-term duration of donor-derived epithelial stem cell viability remains uncertain [19,84−86]. In fact, some studies note rapid disappearance of transplanted cells in limbal allografts or cultured LESC; yet, in many cases, the ocular surface remains healthy long-term [19,87]. Whether donor LSC survival is sustained or activation of resident recipient stem cells occurs on reestablishment of niche signals has yet to be determined. In any event, if the LESC grafts eventually fail, repeat transplants can be made successfully, as a study in burn patients has shown [88].

Tissue-engineered stem cells from noncorneal sources as an alternative to limbal epithelial cells

Transplantation of LESC-enriched cultures faces some problems related to limited number of possible cell passages, allograft rejection risk and disease transmission, as well as shortage of donor corneas in many countries [89]. This necessitated search for alternative cell types that are able to differentiate into corneal epithelial cells and to restore corneal surface. The tested cell sources include cultured oral mucosal epithelium, hair follicle, conjunctival and epidermal epithelium, amniotic epithelial cells, umbilical cord lining epithelium, mesenchymal stem cells (MSCs) from adipose tissue, bone marrow, orbital fat, and immature dental pulp [2,90−92].

Nakamura et al. [78] pioneered the idea of using cultured oral mucosal cells expanded on denuded amniotic membrane in autologous grafts to reconstruct rabbit corneal epithelium. The resultant epithelial cells showed expression of keratin 3 (but not keratin 12) and the formation of a 5−6 layered−stratified epithelium with desmosomes, hemidesmosomes, and tight junctions. Subsequently, autologous-cultured oral mucosal epithelial constructs have been used clinically, usually for treating chemical burns. The success rates of their transplantation were slightly lower than with cultured LESC [2,91].

Most other abovementioned cell types have only been examined in preclinical models with varying success [9,19,64,93,94]. More recently, embryonic and induced PSCs have been differentiated into corneal epithelial cells. Because they can be made autologously, propagated in large quantities, and are bankable; they may become a promising source of limbal cells for transplantation in LSCD of various etiologies [95−99].

Stromal stem cells

Cells expressing Pax6 and ABCG2 progenitor markers as well as MSC markers and capable of differentiating into corneal keratocytes in vitro have been isolated from human limbal stroma [100]. Such stromal stem cells are multipotent and fulfill the International Society of Cellular Therapy criteria for MSC [101]. Similar MSCs have also been found in the central corneal stroma [102]. Corneal stromal stem cells have been used for stromal engineering in vitro [100]. In a lumican-null mouse model of corneal opacity, intrastromal injection of isolated human stromal stem cells was able to restore transparency [100]. Importantly, in a corneal wound model, injection of human stromal stem cells from limbal biopsies prevented fibrotic scar formation, with inhibition of neutrophil migration into the wounded stroma and preservation of normal structure of the stromal extracellular matrix [103,104]. Exogenous MSCs in vivo were also able to significantly reduce stromal neovascularization, corneal opacity, and inflammation in alkaline burn and penetrating injury models [105,106]. The use of compressed collagen as injected cell matrix was shown to enhance their antiscarring ability [107]. These data hold promise for the future clinical use of corneal stromal stem cells and other MSCs for the treatment of burns and reduction of stromal fibrosis. An alternative source for future stromal cell transplantation is represented by keratocytes derived from induced PSCs [108].

Endothelial stem cells

Unlike rabbit cells, human corneal endothelial cells (CECs) can hardly be expanded in vivo or in vitro [109,110]. The existence of human corneal endothelial stem cells was hypothesized for a long time but only recently received experimental corroboration. Expanded neurosphere-forming cells could be incorporated into the endothelial layer in a model of CEC deficiency [109]. Slow-cycling endothelial cells expressing stem cell

markers Oct3/4, Wnt, Pax6, SOX2, SOX9, and with elevated telomerase activity were observed at the corneal periphery [111–113]. Recently, the identification of neural crest–derived progenitors from corneal endothelium of normal subjects and those with Fuchs endothelial corneal dystrophy has been reported. These cells expressed stem cell–related genes as well as neural crest marker genes and were able to differentiate into CECs with typical transcellular resistance [114].

To enhance corneal endothelial repair, other progenitor cells were tested, including MSCs, from umbilical cord blood [115]. As an alternative source for CEC transplantation, such cells can be obtained by in vitro differentiation of human PSCs. As corneal endothelium is derived from neural crest stem cells, such cells could also be used to generate autologous endothelial substitutes [116]. The availability of corneal endothelium differentiated from embryonic or induced PSCs and expressing a set of endothelial markers has been reported [117,118]. Such cells could restore transparency in the eyes of rabbits with corneal endothelial dysfunction [119]. In summary the ability of producing CECs from human PSC-derived neural crest cells provides a possibility to generate endothelial replacements suitable to treat corneal edema and wound-related complications of corneal surgeries.

Conjunctival epithelial stem cells

The conjunctival epithelium, like the corneal epithelium, undergoes constant renewal. It is still unclear which regions of the conjunctiva harbor stem cells, including the fornix, palpebral, and mucocutaneous zones. Slow-cycling cells with higher proliferative capacity were demonstrated in these regions [65,120,121]. Conjunctival stem cells can differentiate into either a mucin-secreting goblet cell or epithelial cell. Interestingly, clusters of corneal epithelial cells residing ectopically in human conjunctival epithelium have been described [122]. The field of conjunctival stem cells as another stem cell source for ocular surface epithelia needs to be expanded.

The bioengineered cornea

The idea of a bioengineered cornea has arisen with corneal equivalents being reconstructed from corneal cell lines. Griffith et al. [123] reconstructed a human cornea from immortalized cells. It was reported that the resultant corneal equivalent behaved similarly to a normal cornea in terms of morphology, transparency, ion and fluid transport, and gene expression. However, corneal bioengineering to produce replacement implants for wounded or diseased human corneas is still at the investigatory stage and needs significant further development in order to generate a corneal equivalent with the same tensile strength, transparency, and durability as a normal cornea. The ideal bioengineered implant is likely to be a composite of biomaterials and pertinent cell types, to provide a transparent, flexible, but strong biocompatible implant, which can withstand surgical procedures, as well as normal day-to-day mechanical stresses. It may also need to provide a compatible scaffold for eventual repopulation by host's epithelial cells. Composite constructs using a combination of limbal epithelial and stromal cells showed superior differentiation and biological properties [124,125]. A recent report described a full corneal equivalent consisting of acellular porcine corneal matrix with seeded human limbal epithelial cells and endothelial cells differentiated from ESCs. This construct was reportedly repopulated by stromal keratocytes within 3 weeks providing a complete corneal equivalent [126]. At 8 weeks, after transplantation to rabbits, the grafts remained transparent with some peripheral neovascularization. The graft morphology resembled that of a normal cornea and the cells expressed their typical markers. This exciting result holds significant promise for the emergence of bioartificial corneal equivalents suitable for human use in the near future. Advances in stem cell differentiation and graft engineering should help overcome technical problems and accelerate translation into clinic.

Retinal progenitor cells

It has been extensively reported that stem cells exist in the retina of fish and amphibians and that these cells add to the retina throughout their lifetime [127]. Furthermore, these cells, which are located at the ciliary–retinal interface, are also able to regenerate a complete retina, including retinal pigment epithelium (RPE), under appropriate experimental conditions [128]. By contrast the neural retina and RPE in mammals are primarily developed by the early postnatal period and show no evidence of the adult regeneration observed in fish and amphibians [127,129].

Interestingly, retinal progenitor cells were not identified in mammals until 2000 [130,131] and were located at the ciliary marginal zone, similarly located as the retinal progenitor cells of fish and amphibians. Retinal progenitors represent only about 0.2% of pigmented cells in the ciliary margin and display many of the properties associated with stem cells. They are multipotent, self-renewing, and proliferating and express the neuroectodermal marker nestin. Other potential markers can include CD133, CD15, Notch, Numb, and FGFR4 [132]. Retinal progenitor cells can clonally proliferate in vitro to form spherical colonies of cells that exhibit differentiation markers for a variety of cell types, including photoreceptors, intermediate neurons, and Müller glia [130,133]. Thus, it would appear that these progenitors have the potential, given the right environment, to be engineered into the morphological and functional layers associated with the retina.

To date, the primary challenge in the use of retinal progenitors is their limited number and their difficult isolation. To circumvent these issues, investigators have used brain-derived neural progenitor cells [134,135], embryonic retinal progenitor cells [136], bone marrow–derived stem cells [137], and ESCs from the inner mass of the mouse blastocyst [138].

Müller stem cells

During the development of the neural retina, Müller cells are generated late in neurogenesis and are not considered to be neuronal progenitors. However, when injury occurs, Müller cells display features of neural stem cells; they self-renew and generate neurons, glia, and retinal neurons [139]. Roesch et al. have found a subset of Müller cells expresses progenitor genes such as Car2, Dkk3, Chx10, and Pax6 [140]. Studies have suggested that the neural stem cell properties of Müller cells typically remain dormant in the normal adult retina [141]; however, during injury activation of the Wnt and Notch pathways act in concert to regulate the stem cell properties of Müller cells [141]. Müller stem cells migrate to the damaged retina layer and can differentiate into neurons [129,142]. Furthermore, it appears from studies in the chick that Müller glia have the potential to become neurogenic retinal progenitor cells [143].

Retinal pigment epithelium stem cells

The RPE cell maintains its proliferation only during the early stage of embryonic development and then remains nonproliferative throughout life. However, human RPE cells can proliferate during pathological conditions, which suggests RPE plasticity during adulthood. Salero et al. have reported a subpopulation of adult RPE cells express stem cell features that can be self-renewed and exhibit multipotency, produce new RPEs or cells with neuronal phenotype [144].

Nonocular stem cells

There are currently many ongoing clinical trials using nonocular stem cells which aim to test the safety and efficacy of transplanting cells in the eye. Ideally, transplanted cells should have a long-term effect by integrating and surviving in the eye. The cells can potentially regenerate damaged retinal vasculature and restore retinal neurons. Also transplanted cells should have limited proliferative potential after integration into the eye to minimize the risk of adverse effects such as teratoma formation in the eye.

Induced pluripotent stem cells (iPSCs)

In 2006 Takahashi and Yamanaka reported that mouse fibroblasts could be "reprogramed" by the use of transcription factors into cells with pluripotency similar to ESCs, and they call these cells induced PSCs (iPSCs) [145]. In the following year, Takahashi et al. and Yu et al. demonstrated independently that human somatic cells were able to be reprogrammed into iPSCs by using different cocktails of transcription factors; they are called: Yamanaka Cocktail (Oct3/4, Sox2, Klf4, and c-Myc) [146] and Thomson Cocktail (Oct3/4, Sox2, NANOG, and LIN28) [147]. They all reported that iPSCs were able to differentiate into all three germ layers and subsequently differentiate into any adult cells.

Because iPSCs can be derived from somatic cells of a donor, such as skin or fibroblast, therefore they are easily accessible and plentiful. Moreover, they can be autologous cells and thus the recipient may not require immunosuppression when administered. iPSCs provide a promising donor source for cell therapy in retinal regeneration (Fig. 60.2). In 2013 the world's first clinical trial involved in the use of iPSCs-derived RPE cells was conducted in age-related macular degeneration (AMD) patients (http://www.riken.jp/en/pr/press/2013/20130730_1/).

Embryonic stem cells/iPSCs in retinal regeneration

Generating retinal pigment epithelial from embryonic stem cells/iPSCs

Currently, there are two main methods to differentiate iPSCs to RPEs, either the use of floating embryoid body-like culture or adherent culture technique. First, fibroblast iPSC amplification is performed by clump passage, followed by differentiation, and mechanical removal of the pigmented colonies for RPE purification [148,149]. The second, more commonly used method uses pluripotent cells that are amplified by clonal propagation using myosin inhibitor (blebbistatin) followed by spontaneous differentiation of pigmented colonies, and then, two rounds of passage to enrich for RPE cells [150].

Various molecules or compounds are used in cell culture to facilitate the differentiation process of iPSC-derived RPEs. In some studies, fibroblast growth factor (FGF) was removed from the culture condition to allow spontaneous differentiation of iPSC-derived RPEs [151]. Other studies supplemented the culture media with retinal differentiation-inducing factors such as inhibitors of Wnt, Nodal, and BMP-4 pathways [152]. In addition, other growth factors or compounds, such as insulin growth factor 1 (IGF-1), retinoic acid (RA), activin A, nicotinamide,

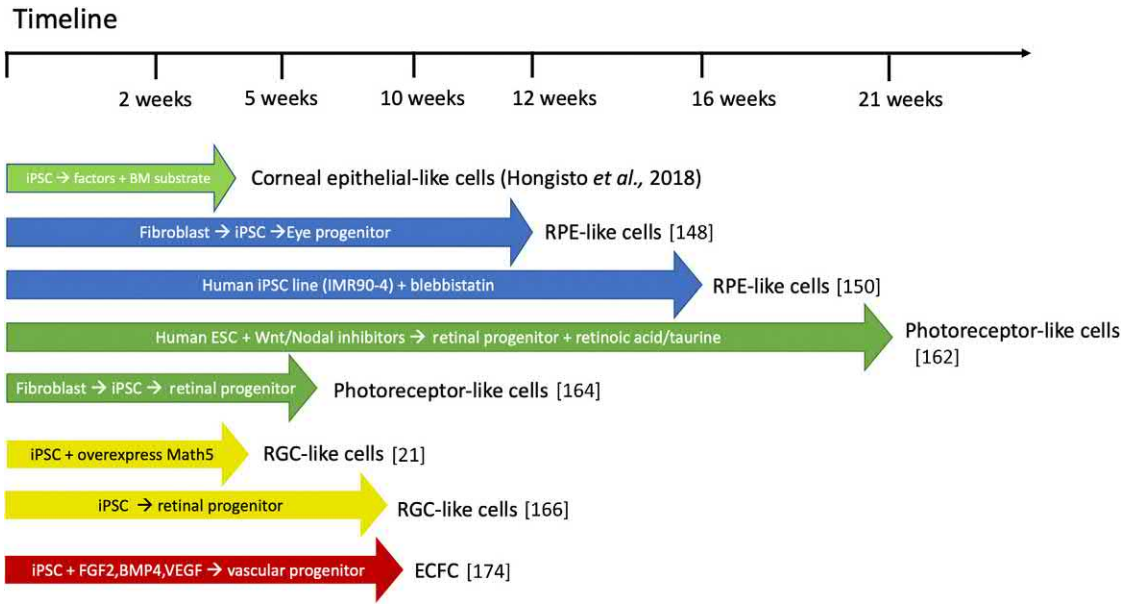

FIGURE 60.2 Differentiation timeline of iPSC/ESC into retinal cells (RPE, photoreceptors, RGC, and ECFC). *ECFC*, endothelial colony-forming cell; *ESC*, embryonic stem cell; *iPSC*, Induced pluripotent stem cell; *RPE*, retinal pigment epithelium; *RGC*, retinal ganglion cell.

SB431542, B27, and N2, have been shown to induce RPE fate [153–155].

In vitro, iPSC-derived RPE cells have been shown to express key RPE markers such as Mitf, OTX2, bestrophin 1 (BEST1), ZO1, PEDF, LRAT, PEML17, and CRALBP; in addition, they have been shown to exhibit essential phenotypic functions, including phagocytosis of photoreceptor outer segments, ion transport, and VEGF secretion [150,151,154,156–158].

In a RPE degeneration rodent model, human iPSC-derived RPEs injected into the subretinal space showed phagocytosis of photoreceptor outer segments and maintained long-term visual function [157]. Similar results by Li et al. demonstrate that subretinal injection of human iPSC-derived RPE cells integrate into the host RPE and improve visual function in recipient mice; importantly, no evidence of tumor formation was observed up to 6 months [159]. These studies provide evidence for the safety and efficacy of iPSC-derived cells in clinical trials.

Of note, a trial using human iPSC-derived RPE to treat exudative AMD has been done in 2017 [160]. The study assessed the feasibility of transplanting a sheet of RPE cells differentiated from iPSCs in a patient with neovascular AMD—at surgery the neovascular membrane was removed and transplantation of the autologous iPSC-derived RPE cell sheet under the retina was performed. At 1 year after surgery the transplanted sheet remained intact, best corrected visual acuity had not improved or worsened, and cystoid macular edema was present. A second trail used ECS-derived cells for individuals with Stargardt's macular dystrophy (age >18 years) and individuals with atrophic AMD (age >55 years). Three dose cohorts (50,000, 100,000, and 150,000 cells) were treated for each eye disorder. Transplanted patients were followed up for a median of 22 months by the use of serial systemic, ophthalmic, and imaging examinations. There was no evidence of adverse proliferation, rejection, or serious ocular or systemic safety issues related to the transplanted tissue. A total of 13 (72%) out of 18 patients had patches of increasing subretinal pigmentation consistent with transplanted retinal pigment epithelium. Best corrected visual acuity, monitored as part of the safety protocol, improved in 10 eyes, improved or remained the same in 7 eyes and decreased by more than 10 letters in 1 eye, whereas the untreated fellow eyes did not show similar improvements in visual acuity. The results suggested that hESC-derived cells could provide a potentially safe new source of cells for the treatment of various unmet medical disorders requiring tissue repair or replacement [161].

Generating photoreceptors from embryonic stem cells/iPSCs

Several methods are used to generate photoreceptors from ESCs/iPSC. Normally, ESCs/iPSCs are derived into retinal progenitor cells in 35 days by blocking Wnt and Noda pathways, then after 170 days, only 20% of the progenitors become photoreceptor precursors. Retinoic acid (RA) and taurine were used to promote maturation of photoreceptors by the expression of rhodopsin and opsin, and this process normally takes 130 and 200 days [162]. A

modified protocol to generate photoreceptor precursor from iPSC by combining Matrigel-coated culture dishes with the addition of Noggin, DKK-1, IGF1, Lefty A 3,3′,5-triiodo-L-thyronine (T3), FGF, RA, taurine, Shh, and Activin A, greatly shortens the process [163,164].

Few transplantation studies were published using human iPSC-derived photoreceptors. One of the studies showed that a limited number of donor iPSC-derived photoreceptors migrate into the outer nuclear layer (ONL) by subretinal injection [165]. In contrast, injection of iPSCs into the vitreous appeared less effective at promoting outer retinal integration [166]. Tucker et al. identify disease-causing USH2A mutations in an adult patient with autosomal recessive retinitis pigmentosa [167]. Induced PSCs (iPSCs) were generated from the patient's keratinocytes and were differentiated into multilayer eyecup-like structures with features of human retinal precursor cells. Transplantation into 4-day-old immunodeficient Crb1 (−/−) mice resulted in the formation of morphologically and immunohistochemically recognizable photoreceptor cells, suggesting that the mutations in this patient act via postdevelopmental photoreceptor degeneration. Wiley et al. developed clinically compatible methods for manufacturing photoreceptor precursor cells from adult skin in a Good Manufacturing Practice (GMP) environment [168]. They obtained biopsies from 35 adult patients with inherited retinal degeneration and fibroblast lines were established. Patient-specific iPSCs were then generated, clonally expanded and validated. Postmitotic photoreceptor precursor cells were generated using a stepwise GMP-compliant 3D differentiation protocol. Transplantation into immune-compromised animals revealed no evidence of abnormal proliferation or tumor formation suggesting that future human trials maybe safe and efficacious using patient-specific photoreceptor cell replacement in humans. Gagliardi et al. showed that the cell surface antigen CD73 was exclusively expressed in hiPSC-derived photoreceptors by generating a fluorescent cone rod homeobox (Crx) reporter hiPSC line using CRISPR/Cas9 genome editing [169]. They demonstrated that CD73 targeting by magnetic-activated cell sorting was an effective strategy to separate a safe population of transplantable photoreceptors. $CD73^+$ photoreceptor precursors were isolated in large numbers and transplanted into rat eyes and showed capacity to survive and mature in close proximity to host inner retina in a model of photoreceptor degeneration. Their results may facilitate future clinical translation. Since single cell injection typically results in poor cell survival and integration posttransplantation, scaffolds can be used to promote donor cell viability, control cellular polarity, and increase packing density [170]. A challenge has been developing suitable scaffolds that can be used to deliver stem cell–derived photoreceptors in an ordered columnar orientation (i.e., similar to that of the native retina). Obstacles to refinement of polymer scaffolds include difficulties in controlling the microstructure of biocompatible substances in three dimensions. Thompson et al. showed that two-photon polymerized acrylated poly(caprolactone) (PCL) scaffolds successfully supported human iPSC–derived retinal progenitor cells in vitro. Subretinal implantation of cell free scaffolds in a porcine model of retinitis pigmentosa did not cause inflammation, infection, or local or systemic toxicity after 1 month supporting the utility of PCL scaffolds for retina cell delivery.

Generating retinal ganglion cells from embryonic stem cell/iPSCs

Different from the photoreceptor that has a single synapse to interneurons, transplanted retinal ganglion cells (RGCs) not only have to integrate into the retinal ganglion layer but also develop long axons that migrate to the optic nerve. This poses a great challenge for replacing damaged RGCs in cell therapy. Nevertheless, progress has been made in generating cells with RGC features from stem cells. Fibroblast-derived iPSCs were induced by addition of DKK1, Noggin (DN), DAPT, and overexpression of Math5 and then differentiated into RGC-like cells. These cells display long synapses and express RGC gene patterns; moreover, transplanted iPSC-derive RGC-like cells into mouse vitreous were able to survive but rarely engraft into the normal host retina [171]. In a different study, iPSCs were induced into neural progenitors with neural induction factors, including N2, B27, insulin, transferrin, sodium selenite, fibronectin, FGF, and Noggin. Then, neural progenitors were cultured in conditioned medium from E14 rat retinal, and a subset of cells expressed mature RGC markers. Moreover, the study found that a subset of iPSC-derived RG-like cells innervate into the mouse superior colliculus ex vivo [166].

Generating hematopoietic/vascular progenitors (CD34/endothelial colony-forming cells) from iPSC

iPSCs are also used to differentiate into vascular progenitor cells and explored as potential cell therapy. Fibroblast-derived human-iPSCs under feeder-free endothelial culture condition give rise to multipotent $CD34^+$ $CD45^+$ hematopoietic progenitors [172]. Cord blood–derived iPSCs differentiate into vascular progenitors that express endothelial/pericytic markers ($CD31^+$ $CD146^+$); moreover, systemic or intravitreal injection of these cells enhanced repair of damaged retinal blood vessels in an ischemic retinopathy model [173]. Prasain et al. generated endothelial colony-forming cells (ECFCs) from human cord blood–derived iPSCs that showed the capacity to form vessels and repair ischemic mouse retina and limbs;

moreover, these cells lack teratoma formation potential [174].

Bone marrow stem cells

Although not resident in the eye, it appears that bone marrow stem cells (BMSCs) play an important role in retinal homeostasis and repair. The heterogeneity of the BMSC population promotes differentiation into various cells. There are two classes of BMSCs that are commonly studied; MSCs and hematopoietic stem cells (HSCs).

Mesenchymal stem cells

MSCs are fibroblast-like cells with a capacity to replicate and differentiate into osteoblast, adipocytes, and bone marrow stromal cells [175]. In bone marrow, MSCs constitute less than 0.1% of the total cells [176]. Yet, they are heavily explored in cell therapy credited by their ability to quickly expand in culture condition while retaining their multilineage potential. Furthermore, MSCs can be administrated intravenously and appear to be safe in clinical trials.

MSCs can be harvested from bone marrow, umbilical cord blood, adipose tissue, placenta, Wharton's Jelly, dental pulp, liver, and heart [177]. Expression of surface markers is used to identify MSCs and include CD105, CD73, stromal antigen 1, CD44, CD90, CD166, CD54, and CD49. MSC is also characterized by the absence of hematopoietic cell (CD14, CD45, CD11a, and CD34), erythrocytes (Glycophorin A), and platelet (CD31) markers [177].

MSCs have been reported to contribute to tissue regeneration through at least three different mechanisms. First, the primary mechanism involves paracrine trophic effects in vitro and in vivo. These paracrine trophic factors include neuroprotective and angiogenic factors such as ciliary neurotrophic factor, bFGF, VEGF, and TGF-β [178]. In pathological conditions such as hypoxia, the production of paracrine trophic factors is further enhanced. In addition, new paracrine trophic factors have been packaged in extracellular vesicles that are secreted by the MSCs [179]. Secondly, MSCs are able to differentiate into damaged tissues [177]. Lastly, MSCs have immune-modulating capacity including immunosuppression and features similar to T-regulatory cells with ability to produce cytokines such as IL-10, IL-17, TGF-β, LIF, soluble HLA, and IL-1 receptor antagonist [180]. Hence, MSCs can be potentially used in treatments for autoimmune diseases, such as Crohn's diseases and multiple sclerosis [181].

Mesenchymal stem cells for cell therapy in the eye

The in vitro study performed by Duan et al. has shown that bone marrow–derived MSCs can be differentiated into cells with RPE features [182]. Subretinal injection of bone marrow–derived MSCs has shown direct integration into the RPE layer and prolongs the survival of photoreceptor [183] and ameliorates the retinal degeneration in animal models [184]. Intravitreal injection of MSCs has been explored in preclinical studies. In an animal model of diabetic retinopathy, intravitreally injected bone marrow–derived MSCs integrate into the inner retina and improve visual function [185]. In an animal model of retinal ischemia-reperfusion injury, bone marrow–derived MSCs administrated intravitreally have shown a protective effect on damaged ganglion cells [186]. In addition, intravitreal injection of MSC into a laser-induced retinal damaged rat model that mimic retinal neovascularization has shown that MSC can migrate into the neural retina, especially in the ONL, inner nuclear layer, and ganglion cell layer [187]. Systemic administration of MSCs preserves photoreceptors, reduces retinal vascular leakage, and improves visual acuity in an animal model of retinal degeneration [188]. Locally and systemically injected bone marrow–derived or adipose-derived MSCs differentiate into cells with photoreceptor and RPE features in an animal model of hereditary retinal dystrophies as well as in mechanical and chemical impaired RPE rat models [137,188–190]. However, it is still unclear whether the observations resulted by differentiation of transplanted MSCs or fusion of MSCs with preexisting photoreceptors [191].

Hematopoietic stem cells/CD34$^+$ cells

Bone marrow has the highest concentration of HSCs, and most of the HSCs can be isolated on the basis of their surface markers, the most common marker is CD34 [192]. Besides bone marrow, CD34$^+$ cells are also found in adipose tissue, umbilical cord blood, and fetal liver [177]. CD34$^+$ cells are capable of self-renewal and responsible for hematopoietic reconstitution in the irradiated host [192,193]. Since then, CD34$^+$ cells were largely explored in clinical testing to repopulate hematopoietic lineage cells [177]. In 1997, Asahara et al. have found that CD34$^+$ cells enriched from human peripheral blood have the ability to on endothelial linage capability characteristics and are capable of being recruited to ischemic sites; moreover, they participate in tissue regeneration and angiogenesis [194]. This suggested that CD34$^+$ cells can mobilize from the bone marrow and be recruited to the injured tissues via blood circulation.

Hematopoietic stem cells/CD34$^+$ and retinal disease

Intravitreal and systemic infused human CD34$^+$ cells enriched from peripheral blood of healthy donors have shown to integrate into the damaged retinal vascular in

diabetic retinopathy and ischemia-reperfusion mouse models, where cells from the diabetic donor were unable to integrate into damaged vasculature [195]. The migratory defect of diabetic $CD34^+$ cells was due to reduced phosphorylation and intracellular redistribution of vasodilator-stimulated phosphoprotein, an actin motor protein for cell migration [196]. In addition, upregulated of the HSC quiescence factor TGFβ-1 in diabetes also contributes to hampering the migratory function of the $CD34^+$ cells [197]. Long-term diabetes can lead to changes in hematopoiesis, in which more bone marrow–derived proinflammatory monocytes and fewer reparative circulating endothelial progenitor cells (EPCs) are migration into the circulation [198]. Moreover, circulating EPCs are trapped in the bone marrow niche in diabetes [199]. Lack of reparative circulating EPCs in diabetes may contribute to the development of retinal degeneration.

A subpopulation of bone marrow derived progenitor cells expressing CD14 was discovered to have vascular reparative function as $CD34^+$. Unlike $CD34^+$, diabetic CD14 appears to retain the robust homing capacity to the damaged retinal vasculature [200]. Moreover, coadministration of MSC with diabetic $CD34^+$ enhanced the migration and colocalization of $CD34^+$ within the retinal vasculature [200]. The study supports the notion that combinations of cells may serve as an enhanced strategy; moreover, it provides evidence for the paracrine effect of MSCs and their ability to enhance the vascular function of defective diabetic $CD34^+$ cells.

Cell-specific gene RPE65 promotes the fate determination of bone marrow–derived stem cells into RPE-like cells for tissue repair. Systemic delivery RPE65 reprogrammed HSCs regenerate the RPE layer in an RPE ablation mouse model (sodium iodinate) [201] and in Sod 2 knockdown mouse model that has similar pathological features of AMD [202], and importantly, restored the visual function of these animals. Besides preserving damaged retinal vasculature, intravitreally injected bone marrow–derived lineage-negative HSCs contain endothelial precursors that have been shown to rescue photoreceptors without direct integration in nonischemic murine neurodegenerative diseases [203]. The indirect preservation of retinal neurons is, in part, via the neurotrophic effect of injected EPCs. Fukuda et al. showed that intraocular-injected EPCs recruit neuroprotective macrophages by secreting neurotrophic factors [204]. A similar neuroprotective function by paracrine factors was observed with bone marrow–derived $CD34^+$ cells [205].

EPCs such as $CD34^+$ cells represent a heterogeneous population of progenitor cells. The nomenclature is unable to match the increasing complexity of the cell. Moreover, murine stem/progenitors are characterized by different surface markers than humans and make it difficult to directly compare animal studies to human studies [206]. Therefore, a consensus was made in the field to give a more accurate terminology to define EPCs. In general, there are two main subtypes of EPC. One subtype is circulating angiogenic cells (CACs) or myeloid angiogenic cells (MACs) (Fig. 60.3). They represent a population of cells of hematopoietic origin that express CD45, CD14, and CD31 but are negative for CD146 and CD34. CACs/MACs both promote angiogenesis in a paracrine manner [207].

Endothelial colony-forming cells

Another subtype of EPCs is ECFCs (Fig. 60.3). This endothelial progenitor population has high proliferative capacity in vitro and capable of direct incorporation into

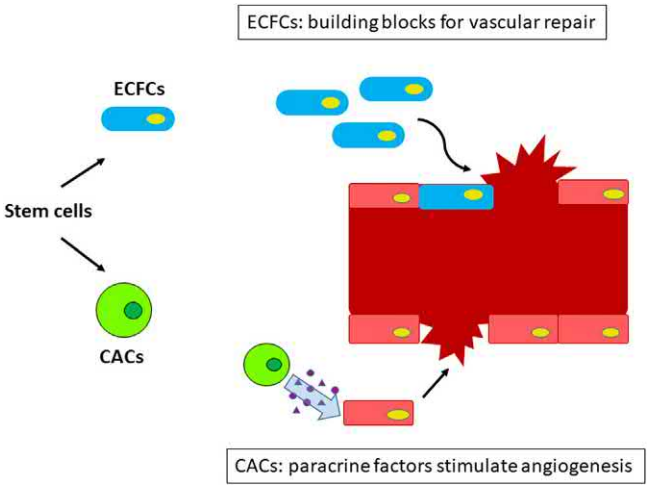

FIGURE 60.3 Stem cell–induced endothelial progenitors participate in retinal vascular repair. CACs release paracrine factors as stimulants of angiogenesis. ECFCs are the bona fide endothelial progenitors capable of direct incorporation into the injured endothelium. *CAC*, Circulating angiogenic cell; *ECFC*, endothelial colony-forming cell.

injured endothelium or de novo blood vessel formation in vivo [207–210]. ECFC represent the cell population with the expression of CD31, CD105, and CD146 and negative for CD45 and CD14 and these cells are vascular wall derived. Although these cells express CD34, the expression level may decline during in vitro expansion [207].

Administration of ECFC, a bona fide endothelial progenitor, has been demonstrated in preclinical studies to promote vascular repair in ischemic tissues, such as in myocardium [211], brain [212], hind limb [213], and kidney [214]. Recently, the vascular repair and vasoregenerative function of ECFCs were also examined in the murine ischemic retina [174,215,216]. In preclinical studies, intravitreal and systemic delivery of ECFCs has shown equal efficacy in retinal vascular repair; moreover, intravitreal delivery of ECFCs did not evoke an inflammatory response in the mouse [216]. These studies suggest the remarkable potential of these cells for clinical trials.

Therapeutic uses of ECFCs are primarily focused on harnessing their blood vessel-forming capacity. Recent studies have shown that ECFCs can exert trophic functions in support of engraftment of MSC [217]. Besides, CD44 cells, a subclass of ECFCs can promote vascular and neuronal protection by secretion of trophic factors in animal models of retinal diseases [215].

The potential for stem cells in ocular repair and tissue engineering

The realization of the existence of undifferentiated cells, which self-renew and show plasticity, in adult tissues offers great potential in the treatment of both ocular and nonocular diseases. It is now becoming evident that both intraocular and extraocular stem cells can play a critical role in the maintenance of ocular tissues. Furthermore, the plasticity of these cells means that they can be engineered to repair injured or diseased ocular tissues. Corneal LSCs are now routinely used in the restoration of the injured ocular surface, and BMSCs hold great promise in the repair of retinal damage. Ocular stem cells are an ideal starting point for tissue engineering of ocular tissues and offer a means of therapeutic intervention in a variety of pathologies involving cell/tissue loss (e.g., AMD, retinitis pigmentosa, glaucoma, and diabetic retinopathy). The future holds the promise of stem cells being used for gene delivery to correct genetic abnormalities in specific ocular tissues.

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Chapter 61

Corneal replacement tissue

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Introduction

Corneal blindness is the fourth leading cause of vision loss worldwide. It is caused by a variety of pathologies, including trauma or infection of the corneal surface, as well as congenital diseases that lead to degeneration of corneal cells [1]. Trauma and infection are the most common cause of corneal blindness in developing countries [2], whereas corneal degeneration is predominant in Europe and the United States [3]. Regardless of causation, the end stage of all corneal diseases is characterized by irreplaceable tissue loss. In these cases, patients have no therapeutic option other than undergoing surgery to replace part of their damaged cornea with donor corneal tissue (corneal transplantation, also called corneal allograft or keratoplasty) [1] (Fig. 61.1).

The first corneal transplantation was successfully performed in 1905, by Eduard Zirm, on a blind patient suffering from lime burns [4]. Since then, corneal transplantation has become one of the most frequent types of transplantation performed worldwide, with thousands of procedures performed each year. As a reference, in 2012, 185,000 corneal transplants were performed globally [5]. Due to the immune privileged environment of the eye, corneal transplants are quite successful, with an estimated graft survival rate of approximately 80%−90%, at 1 year after transplantation [6−8]. Still, a significant number of patients, particularly among those who exhibit inflammation and vascularization of the cornea, show rejection and require a new graft within 5 years of transplantation [9]. Unfortunately, access to good quality corneal tissue at an affordable price remains a significant challenge in many parts of the world. Currently, 12.7 million patients worldwide are waiting for a corneal transplant, without taking into consideration patients in remote rural areas with poor access to eye care [5]. This donor shortage will likely continue due to population growth and a demographic shift toward an aging population.

To address such a challenge, new therapeutic approaches have gained considerable experimental and clinical attention. The use of artificial corneas composed of clear plastic, for example, has increased over the past decade. However, this approach is limited to extremely severe cases due to complications and high host rejection rates [10,11]. The use of biocompatible materials alone or in combination with cells has emerged as a promising alternative. In this chapter, we will describe recent developments of such new therapeutic approaches in the context of different corneal cell types and functions.

Corneal anatomy and structure

The human cornea is a transparent, avascular tissue at the outermost part of the eye. It forms a barrier that protects the eye from damage or infection. The cornea also serves as the primary lensing unit of the eye refracting and focusing light to ensure proper transmission through the pupil and lens onto the photosensitive retina. Maintaining corneal transparency is therefore paramount for visual acuity.

The cornea is composed of three layers from anterior to posterior: epithelium, stroma, and endothelium (Fig. 61.1). The epithelium is separated from the stroma by the Bowman's membrane, while the endothelium is separated from the stroma by the Descemet's membrane (DM). Both the Bowman's and DMs are enriched with various types of collagens and proteoglycans, performing structural and physiological functions to maintain healthy corneal epithelial and endothelial cells, respectively [12]. Recently another distinct layer, termed pre-DM or Dua's layer, was identified in between the stroma and the conventional DM. The exact significance of this membrane awaits further study, but one potential role is to provide additional mechanical strength and support for the DM [13,14]. The cornea is also densely innervated and one of

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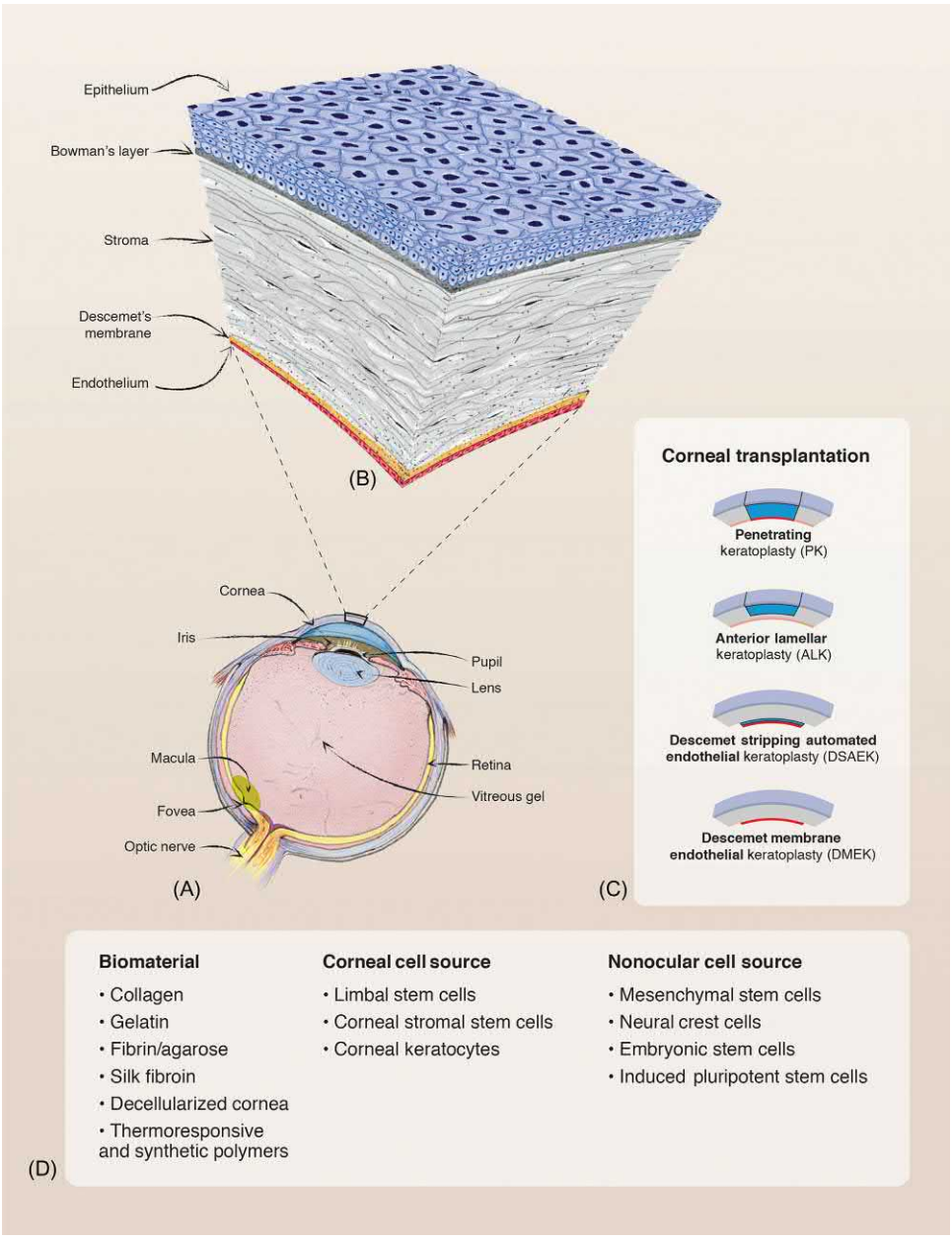

FIGURE 61.1 (A) Representation of the eye indicating the different anatomical structures, including the cornea; (B) schematic of human cornea cornea layers: the outermost stratified squamous epithelium layer with underlying the Bowman's layer, followed by the stroma containing keratocytes, the Descemet's membrane, and the single-layer endothelium; (C) schematic of different types of corneal tissue transplantation: PK: full-thickness corneal transplant replacing the central part of cornea, ALK: when only the stroma is replaced; DSAEK and DMEK: partial thickness corneal transplants used to replace the endothelium layer of the cornea. DMEK is a pure replacement of the endothelium and does not contain any stromal cells; (D) overview of bioengineering approaches for corneal tissue replacement.

the most sensitive tissues in the body. These nerves are essential for sensations of touch, pain, and temperature, as well as for controlling blinking, production of tears, and overall corneal health and structure [15]. Since the cornea does not contain any blood vessels, all nutrients are supplied through diffusion from the tears and the aqueous humor, a clear liquid found in the anterior chamber inside the front part of the eye.

Considering this highly organized structure, the development of an adequate replacement for cornea tissue should encompass strategies that effectively recapitulate both the correct corneal structure and function. For example, any bioengineered corneal tissue replacements should withstand proper ocular pressure and be transparent. For this purpose, not only cells but also the combinations of cells, small molecules, scaffolds, and proteins are currently being explored at the preclinical and clinical levels.

Epithelium

The corneal epithelium is the most superficial layer of the cornea. Along with tears, it creates a smooth surface that allows the refraction of light, protects the eye from environmental insults (microbial and chemical), and transfers water and solutes in and out of the stroma. The corneal epithelium consists of a single layer of basal cells and

TABLE 61.1 Representative markers for corneal cell types.

Corneal epithelial cell	Corneal keratocyte	Corneal endothelial cell
ALDH3A1	ALDH3A1	Na + /K + ATPase
K12	PTDGS	ZO-1
CLU	KLF4	N-Cadherin/Cdh2
PAX6		PITX2
		SLC4A4
		CAR2
		Col4A2
		Col8A2

4−6 layers of stratified, transparent, nonkeratinized wing cells (Table 61.1). The cells of the outermost 2−3 layers are squamous, flattened cells (Table 61.1) that are continually shed and regenerated every 7−10 days. This regenerative capacity depends on adult stem cells located at the border of the cornea and the sclera in a location called the limbus [16]. Under normal conditions or during the healing process following a moderate injury, these limbal epithelial stem cells (LSCs) give rise to transient cells that proliferate, migrate to the center of the cornea, and gradually differentiate to basal and wing cells. These new cells replace the superficial squamous cells at the outer layer of the corneal epithelium [17]. Although an exclusively LSC selective marker remains unknown, certain proteins such as P63a, ABCG2, cytokeratins, and ABCB5 are commonly used to define LSCs [18].

Chronic pathological conditions, such as Stevens−Johnson syndrome, or severe trauma (e.g., chemical or thermal burns) can disrupt the limbal stem cell niche or permanently damage LSCs, causing limbal stem cell deficiency (LSCD) [19]. As limbal stem cells fail to regenerate and maintain the corneal epithelium, new vascular vessels are formed, and conjunctival epithelium invades the cornea resulting in corneal opacity and vision loss. Corneal transplantation is not effective for LSCD as it addresses neither the continuous need for epithelial regeneration nor the risk for early graft rejection due to the invasion of the cornea by conjunctival cells. LSC transplantation, therefore, represents the only promising therapeutic approach for LSCD. Due to the accessibility of corneal epithelium at the outermost layer of the eye, research on LSCD cell therapy has a long history spanning nearly five decades [20].

LSCs can be transplanted either as autologous or allogeneic limbal tissue, or as single cells that have been expanded outside of the body. The first successful limbal transplantations were reported in 1989, in which autologous limbal tissue from the uninjured or less injured donor eye was used on the severely injured recipient eye [21]. The majority of those patients showed sustained regeneration of the corneal epithelium and vision recovery without any complications. These salutary results paved the way for the widespread use of autologous limbal tissue grafts for LSCD, establishing this approach as the standard of care in the United States. Allogeneic tissue grafts are also in use, particularly for patients with bilateral disease, but require long-term immunosuppression and have significant rejection rates after 3−4 years limiting their success and application [22].

To circumvent these challenges, in 1997, autologous limbal stem epithelial cells were expanded in vitro, followed by transplantation into unilateral LSCD patients resulting in the successful restoration of corneal epithelium in patients with moderate to severe LSCD [23]. Developments based on this method have led to the first cell-based treatment (Holoclar) for LSCD caused by burns in Europe [24]. Clinical investigations for Holoclar are still ongoing in the United States and are expected to be completed in 2020.

Using autologous LSCs would work well for unilateral LSCD patients. However, bilateral LSCD patients have limited numbers of LSCs, and alternative sources of cells are required. In 2004 two groups reported that autologous oral mucosal epithelial cells can be cultured and differentiated into corneal epithelial-like cells in vitro, using appropriate scaffolds. When these cells were transplanted into patients with LSCD, 70% of patients showed improvement [25,26]. Studies using allogeneic LSCs have also reported positive outcomes, potentially due to loss of immunogenicity during culture and improved cell retention after transplantation [27].

Unfortunately, using primary cell sources for replacement of the corneal epithelium comes with significant logistical challenges that can preclude their clinical use. Appropriate donor tissue needs to be identified and screened for pathogens. In addition, cells need to be isolated, cultured in vitro, and extensively characterized prior to transplantation to ensure that there is no product variability due to donor or process variants. For instance, it is rather common that primary isolated cells fail to expand in culture due to technical variability or due to the age and disease state of the donor. Thus it would be highly desirable to have an unlimited allogeneic cell source to generate transplantable cells. The use of pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced PSCs (iPSCs), offers such an option. The first study to successfully differentiate corneal epithelial-like cells from human PSCs used limbal fibroblast conditioned media as a means of replicating the conditions of the corneal stem

cell niche [28]. Since then, more than 10 protocols have been published describing the generation of LSCs or corneal epithelial cells from PSCs using various growth factors, undefined or animal-derived components, alone, or in combinations [29].

Regardless of the cell source used (primary or PSC-derived cells), most recent culture protocols include a variety of scaffold materials such as human amniotic membranes, fibrin substrates, and collagens [30]. Of these the human amniotic membrane has been the most extensively tested with a relatively high rate of success. However, the production of clinical-grade amniotic membrane is laborious and expensive and can result in variable stability in vivo, carrying a significant risk for graft rejection [31]. Fibrin, due to the ease of preparation, has been widely tested as an alternative and it is the substrate used in the manufacturing of Holoclar [32]. Recently, cell sheets and contact lenses have gained more attention as they allow for the utilization of FDA approved materials. Questions regarding how to optimize the attachment, alignment, and maintenance of cells in these structures remain, although some initial successes have already been reported using this approach. Indeed, a team in Japan has already initiated tests to treat four patients with a corneal transplant. The transplant will be a 0.05 mm thick cell sheet composed of corneal epithelial stem and progenitor cells derived from iPSCs cultured in a specifically designed laminin E8 matrix [33]. The first patient treated has already shown visual improvements, 1 month after cell transplantation, without any adverse effects [34,35].

Stroma

The stroma is a highly organized structure, crucial for maintaining the transparency and mechanical strength of the cornea. Among the three distinct corneal layers the stroma constitutes the largest part of the cornea (approximately 90% of thickness) (Fig. 61.1). It is mainly composed of extracellular matrix, primarily type I collagen, forming tightly packed parallel collagen fibrils (lamellae). The size and spacing of these fibrils are regulated by the presence of the proteoglycans, as well as by the ratio of the different types of collagen (collagen type I to type V ratio). The corneal stroma also contains a small population of specialized cells called keratocytes [10], (Table 61.1). Keratocytes make up only 3%−5% of the stromal volume and are embedded between the collagen lamellae. Developmentally, keratocytes are derived from neural crest cells (NCCs) and are characterized by the expression of keratan sulfate−containing proteoglycans, such as keratocan and lumican, as well as human aldehyde dehydrogenase 3A1 (ALDH3A1), prostaglandin D2 synthase (PTDGS), and the transcription factor KLF4 [36,37]. The primary function of the keratocytes is to deposit the extracellular matrix protein found in the stroma [38].

Keratocytes are typically nonproliferative. However, upon injury, they begin to divide and secrete extracellular matrix components promoting scar resolution and healing of the cornea [10,39,40]. Intriguingly, keratocytes can also secrete neurotrophic and pro-inflammatory factors, such as IL-8, IL-15, MCP-1, eotaxin, and RANTES, which can promote nerve regeneration in the damaged stroma [41]. During severe injuries, activated keratocytes progressively express vimentin, αSMA, and desmin and become myofibroblast cells. The development and persistence of these myofibroblasts can lead to disorganization of the collagen lamellae, clouding of the stroma, and eventually to permanent scar, fibrosis, and loss of visual acuity [40]. In these cases, alternative approaches are required to reconstitute the proper structure and function of the stroma.

Reflecting its mostly collagenous nature and in contrast to the cell-centric nature of epithelial and endothelial tissue engineering, the main approaches for stromal tissue engineering are scaffold-based. To mimic the collagenous structure of corneal stroma, several studies have used collagen scaffolds, such as collagen hydrogels, sponges, films, and augmented collagen scaffolds [10]. Other materials such as synthetic polymers and silk fibroin, as well as more elaborate methods such as cornea decellularization, have also been explored [10]. Zhang et al., for example, have used decellularized porcine corneas for transplantation in patients with corneal fungal ulcers. More than 70% of these patients recovered their corneal transparency and showed visual improvement [42]. Still, the use of xenogeneic material carries the risk of rejection or allergic reaction [43−45]. A recent intriguing approach that circumvents this risk is the use of 3D printing to deposit recombinant human collagen to create collagen layers that mimic stromal structure. Corneal stromal cells successfully grew and penetrated into these 3D-printed scaffolds, opening up the possibility of more customizable stromal tissue engineering [10].

In recent years the strategies for corneal stroma tissue engineering have expanded to include the combinations of scaffolds with cells. Initial efforts have been focused on the culture of isolated primary keratocytes; however, the isolated cells lost their keratocyte characteristics in vitro [46]. To preserve keratocyte identity, Che et al., cultured the cells on an ultrathin layer of amniotic membrane and developed a multilayer, stroma-like construct that they then transplanted in a rabbit model. Four weeks post-surgery, the implants appeared to be well integrated into the cornea, promoting collagen lamellae formation [47]. Corneal stromal stem cells present in the limbal niche below the basement membrane have also been isolated. These cells can adopt a more mature keratocyte

phenotype in culture and can deposit collagen and other stromal extracellular proteins [48]. When these cells were transplanted in mice with corneal stromal defects, they restored stromal thickness and collagen fibril organization [49]. In addition to corneal cells, multiple sources of mesenchymal stem cells (MSCs) such as bone marrow, adipose tissue, and umbilical cord have been used to generate cells with a keratocyte phenotype [50]. At the clinical level, adipose-derived MSCs from patients were transplanted, alone, or in combination with decellularized human corneal stroma, in patients with advanced keratoconus. No complications were observed after a 1 year follow-up, and corneal transparency was recovered in all patients [51]. Though promising, this approach still has challenges as it heavily relies on autologous sources of cells and donor-derived corneal stroma. Consequently, recent efforts have been focused on generating keratocytes derived from human-iPSC (hiPSC). As an example, Naylor et al. have seeded hiPSC-derived NCCs onto cadaveric human limbal rims. After 21 days in culture the cells acquired a morphological and molecular profile similar to that of native corneal keratocytes [37]. Whether these cells can functionally replace corneal keratocytes, alone or in combination with scaffolds in vivo, remains to be tested.

Endothelium

The corneal endothelium constitutes the innermost layer of the cornea, separating it from the aqueous humor in the anterior chamber of the eye (Fig. 61.1). The endothelium consists of a single layer (approximately 4 μm) of squamous epithelial cells, which are characterized by the distinct expression of ion pumps such as Na + /K + /ATPase and tight junction protein such as ZO-1 (Table 61.1). This layer serves as a barrier to maintain the proper corneal dehydration state via a pump-leak mechanism [52,53].

Loss or dysfunction of the corneal endothelial cells (CECs) creates excess fluid buildup in the cornea, resulting in progressive swelling, stromal disorganization, reduced transparency, and impaired vision [54]. The leading causes of corneal endothelial loss and dysfunction are endothelial dystrophy, trauma/surgery, or chronic anterior uveitis. Fuchs' endothelial corneal dystrophy and congenital hereditary endothelial dystrophy are among the most common corneal endothelial dystrophies caused by genetic mutations and potential environmental factors [55]. Another frequent cause of endothelial dystrophy, pseudophakic bullous keratopathy, is induced by damage to the endothelium during cataract surgery. Diabetes and aging are also significant risk factors for corneal endothelial loss.

Unlike epithelial cells that can repair by proliferation, adult CECs have limited proliferative capacity. Instead, endothelial cells tend to repair by sliding toward the area of injury and enlargement of adjacent cells. Although this mechanism maintains the corneal endothelial barrier after injury, it also reduces endothelial cell density and alters their hexagonal morphology and pumping capacity. When the reduction in cell density reaches a range of 500−1000 cells/mm^2 or less, the only available therapeutic option is partial or complete corneal transplantation [54]. Currently, corneal transplantation for endothelial loss accounts for 60% of all corneal transplants performed in the United States [56].

To overcome the above-described corneal tissue donor shortages, initial efforts have focused on the isolation and ex vivo expansion of primary CECs. The first successful culture of human CECs was reported in 1965 [57]. Since then, several modifications in isolation techniques, extracellular matrix substrates, and culture media have been developed to overcome the cells' limited propagation capacity and the loss of the CEC phenotype in culture [58,59]. For instance, Okumura et al. examined the expression of different laminin isoforms in the cornea and identified laminin-511 and 521 as the predominant laminin forms in adult DM [60]. Using these two laminins as a substrate for the in vitro culture of human CECs, they managed to significantly improve the attachment, survival, and expansion of CECs [60]. Other groups have tried culturing human CECs on human donor−derived DMs as substrates. The advantage of this approach is that it mimics the in vivo corneal environment to achieve proper CEC maintenance and growth [61,62]. The importance of DM in supporting CEC expansion was also confirmed by a recent study conducted in a rabbit corneal endothelial injury model. Through this, it was demonstrated that the absence of DM impaired native CEC migration and cornea regeneration [63].

Despite the advantages, the use of scaffolds complicates delivery into the cornea, necessitating elaborate surgical procedures. A much simpler approach would be to deliver CECs in cell suspension. The injection of cells in suspension would allow simpler manufacturing methods and theoretically be less invasive [64]. However, this approach increases the risk of the removal of injected cells by the flow of aqueous humor, limiting cell survival and engraftment in the cornea. Two methods developed to improve the delivery of cell suspensions into the corneal endothelium include the use of magnetic field guided delivery and the coinjection of cells with a ROCK inhibitor. For magnetic cell delivery, CECs are labeled with magnetic particles and delivered to the appropriate location on the posterior corneal surface with the help of an external magnet [65−67]. In a recent example, CECs were labeled with superparamagnetic nanoparticles and injected in the anterior ocular chamber of adult rabbits with CEC injury. Similarly to native CEC, the injected

cells formed a monolayer and restored corneal transparency without any adverse effects [68]. Alternatively, Okumura et al. used coinjection of CECs with the ROCK inhibitor Y27632 to enhance CEC survival and proliferation. This approach resulted in better engraftment of the cells and improved recovery of corneal transparency in a rabbit model of corneal injury [69]. These salutary effects were confirmed in a monkey model of corneal endothelial dystrophy [69], leading to the initiation of human clinical studies. In 2018 the same group reported the results of transplantation of CECs, in combination with ROCK inhibitor, in 11 patients of bullous keratopathy; more than 80% of the treated patients showed recovery of corneal thickness and improved visual acuity 24 weeks after treatment [70]. This study provided the first clinical evidence that the minimally invasive transplantation of cultivated human CECs can be utilized as a new therapeutic option to treat corneal endothelial dysfunction. At the same time, it raised questions regarding the future development of this approach. These include the need to determine a suitable donor age range for the production of high-quality transplantable CECs, the mechanistic contribution of ROCK inhibitor to the clinical outcomes, the precise dosing of cells to balance efficacy and safety, and the development of a means of tracking the fate of delivered cells after transplantation [71]. In addition, the limited availability of high-quality donor CECs and the limited capacity of the cells to expand in vitro are pausing some significant challenges for the clinical translation of this approach.

For this reason, several groups have been trying to generate CEC-like cells from alternative stem cell sources, such as MSCs, skin-derived cells, corneal stromal cells, and PSCs [72–77]. These studies applied our current understanding of embryonic development of CECs by isolating putative CEC progenitor populations, such as NCCs, from primary cells and differentiating them to more mature CEC-like cells. For example, corneal stroma cells and skin-derived precursors containing NCCs were isolated and cultured in the presence of retinoic acid and GSK 3β inhibitor (Wnt signaling pathway activator) to induce CEC-like cells. These cells showed upregulation of Pitx2, a key transcription factor for the development of the anterior eye segment, as well as upregulation of the CEC markers Atp1a1 and Cdh2. Importantly, the cells exhibited functional characteristics of CECs, such as pump function. When the cells were transplanted using a collagen sheet as a carrier, they restored vision in a rabbit model of keratopathy [72,73]. Using a similar approach, Shen et al. differentiated skin-derived precursors by coculturing with B4G12, an immortalized human CEC line. Within 1 week of differentiation, the cells exhibited marked expression of typical CEC markers. Transplantation of these cells resulted in the successful recovery of corneal thickness and corneal transparency in both rabbit and monkey models of corneal endothelial dystrophy [74].

NCCs can also be generated in vitro from PSCs, offering an unlimited and expandable source of cells for clinical application. Zhang et al. reported the differentiation of human ESCs to periocular mesenchymal precursor cells (POMPs), a subtype of NCCs, by transwell coculture with human corneal stromal cells. These induced POMPs were cultured in conditioned media from lens epithelial cells, and differentiation was confirmed by testing the gene expression of typical CEC markers such as N-Cadherin, FoxC1, and PITX2. To enrich for CEC-like cells, the cells were then sorted and seeded on a decellularized porcine corneal matrix followed by successful transplantation and induction of corneal repair into a rabbit model of corneal dystrophy [78].

To avoid the use of xenogeneic material from decellularized corneas, recent approaches are employing small molecule compounds and xeno-free growth factors to induce the CEC cell fate. Several groups, for example, have shown differentiation of PSCs to NCCs following the inhibition of the TGFβ/SMAD pathway [75–77]. These NCCs were then cultured in media containing PDGF-BB and Dkk2, which induced the cells to acquire the hexagonal appearance and express molecular markers of CEC cells [75,77]. Importantly, the cells could deposit collagen generating an in vitro-generated DM and expressed major components of the corneal endothelial pump function such as Na^+K^+ATPaseα1 [75]. Although promising, the functional aspect of these PSC-derived CEC-like cells awaits further investigation. Some critical future questions include the ability of these PSC-derived CEC-like cells to function as a leaky barrier and maintain the dehydration state of the cornea, as well as the exploration of their in vivo therapeutic potential.

Conclusion

Cornea transplantation is constrained by a lack of donor corneas, leaving many patients with visual impairment and blindness. Parallel advancements in bioengineering and the stem cell fields have significantly progressed our understanding of corneal biology and allowed for the development of novel strategies for corneal tissue replacement. Due to the relative simplicity of their isolation and expansion in vitro, corneal epithelial limbal stem cell transplantation for LSCD treatment has progressed the farthest along the clinical development path. Current success rates are in the range of 60%–80%, and further improvements are expected as we discover specific LSC stem cell markers that allow better isolation methods and use of the cells. In parallel the development of innovative methods to produce 3D tissue-like structures that mimic

the microarchitecture and physiology of the native cornea offers opportunities to reconstruct cornea tissue shape, organization, and function, particularly in regards to corneal stromal repair [11,79]. The recent clinical advancements, along with the rapidly expanding development of methods for generating CEC-like cells from multiple stem cell sources, will also increase the emphasis on endothelium replacement via cell therapy and tissue engineering in the coming years.

The use of PSCs as a starting source to generate corneal cells is also expected to gain traction as new differentiation protocols are developed, and the regulatory path to the clinic is established. One advantage of corneal diseases for cell therapy is the immune-privileged environment in the eye, which is permissive to allogeneic PSC sources for generating corneal cells. Still, some level of immunosuppression regimen is required to prevent cell rejection, and unfortunately some patients are unable to tolerate this regiment for long term. Cell engineering, or a combination of cells with new materials, is bound to play a significant role in addressing this concern.

In addition, the use of stem cells−derived extracellular vesicles (EVs), which are presumably less immunogenic than cells, has recently emerged as an alternative therapeutic option. Several studies have reported the secretion of EVs from corneal cells, such as limbal epithelial cells and mesenchymal stromal cells, establishing their potential function in proliferation and repair in the corneal environment [80,81]. Further investigations are required to explore the significance of these findings and provide clear evidence of in vivo functionality. Finally, one of the key considerations for the translation of cell-based approaches to real world therapies is the complexity of the processes and materials needed to generate the cells or the scaffolds. To have a wider clinical applicability, therefore, it is necessary to develop streamlined robust protocols using xeno-free materials that ensure the manufacturing of these products in safe and effective ways.

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Chapter 62

Retinal degeneration

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Epidemiology of visual impairment and blindness

An estimated 1.3 billion people globally suffer from some form of visual impairment. Of those, 188.5 million people have mild visual impairment, which can be defined as a best corrected visual acuity (BCVA) of worse than 6/12 but at least 6/18 or better in a standard Snellen eye chart test. This means that a person with mild visual impairment needs to stand 6 m from a letter chart in order to read letters that a person with "normal" vision could see at a distance of either 12 or 18 m, respectively. An estimated 217 million have moderate-to-severe impairment (a BCVA of worse than 6/18 but at least 3/60 or better) and 36 million people are blind (with BCVA of worse than 3/60) [1]. The age of onset for visual impairment can vary depending on the underlying disease, but those over the age of 50 are at an increased risk since visual impairment in general is associated with aging [1,2]. The five most common causes of visual impairment are uncorrected refractive errors (e.g., myopia, hyperopia, presbyopia, and astigmatism), cataracts (clouding of the lens), and the retinal degenerative diseases: age-related macular degeneration (AMD), glaucoma, and diabetic retinopathy (DR) [2,3]. Although cataracts can be treated with surgery, limited access to appropriate medical intervention, particularly in lower income countries, means they often go untreated and make cataracts the leading cause of blindness worldwide [4]. In higher income countries where access to surgery is more common, the relative prevalence of blindness due to retinal degenerative diseases such as glaucoma, AMD, and DR increases.

Retinal degeneration involves the progressive loss of specific cell types within the retina. Depending on the disease, different cells are more severely affected than others, yet the net result of retinal degeneration is progressive visual impairment, which may lead to blindness. Although some drugs are available to treat these disease, such as anti-vascular endothelium growth factor (VEGF) treatment for wet AMD and DR or intraocular pressure (IOP)–lowering drugs to treat glaucoma, these medications cannot replace cells that have already been lost due to the degenerative process and often do not provide enough support to rescue cells that are damaged and at risk of dying. Cell-based therapies and tissue engineering have an opportunity to do both upon engraftment in the retina; they may (1) provide trophic support for sick or dying cells and/or (2) functionally replace host cells that have already been lost due to degeneration. The chapter discusses cell-based therapies and tissue engineering approaches to the most common retinal degenerative diseases.

Structure/function of the retina and cell types affected in retinal degenerative diseases

In order to understand how cell-based therapies and tissue engineering can be used to treat retinal degenerative diseases, it helps to understand the basic structure/function of the retina, its cell types, and their role in the visual transduction cascade. The retina is a stratified, light-sensing tissue that resides at the back of the eye adjacent to the choroid layer, which contains vasculature and, beyond that, the sclera, which is the white-colored collagen-containing outer lining of the eyeball. Each layer within the retina contains neuronal cells with specialized functions that work together to convert light signals to electrical ones and relay these signals to the brain. The most distal layer of the retina comprises retinal pigment epithelium (RPE), which is a single layer of hexagonal-shaped, polarized epithelial cells (Fig. 62.1).

The RPE attaches to the choroid through Bruch's membrane and supports the health of adjacent rod and cone photoreceptors through processing of retinoids,

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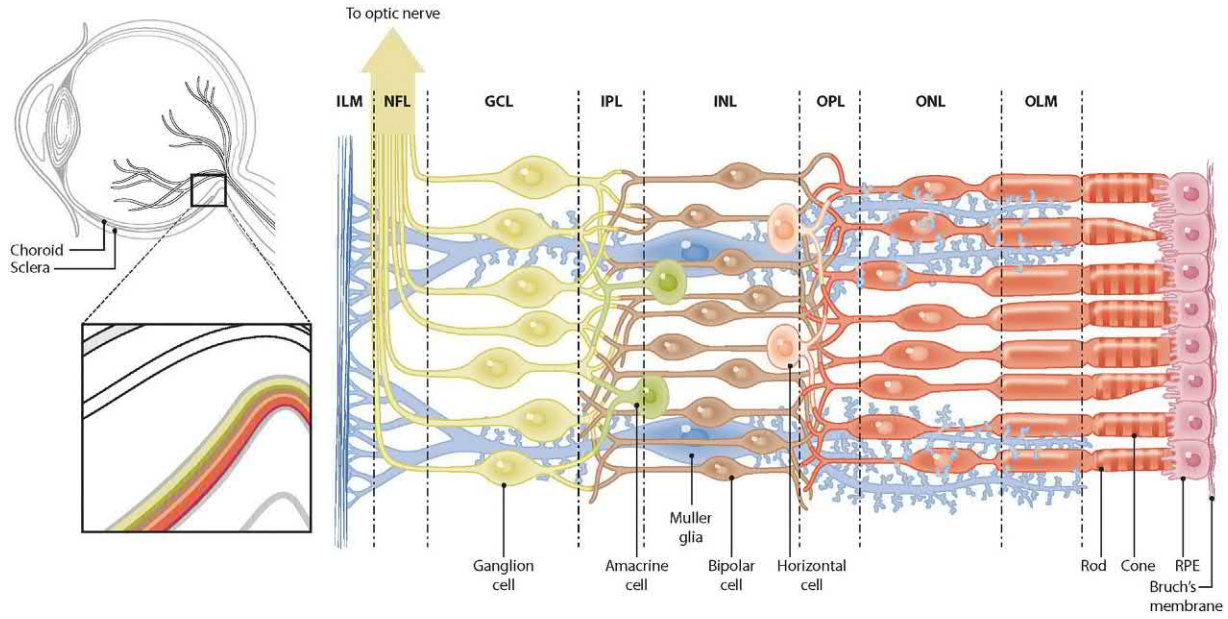

FIGURE 62.1 The eye and the cellular layers of the retina.

absorption of scattered light, ion and fluid transport, and phagocytosis of shed photoreceptor outer segments [5]. Tight junctions between individual RPE cells help form a barrier that works in concert with the blood retinal barrier to maintain the immune privileged status of the eye and regulate ionic exchanges between the circulation and retina. The RPE may also play a more active role in suppressing the immune responses in the eye. It has been found that RPE cells can suppress T-cell activation by altering expression of T-cell activation markers such as CD69 and CD25, and secretion of IL-10, induce a regulatory T-cell phenotype, and trigger T-cell apoptosis [6–9].

As the main light-sensing cell type within the retina, photoreceptors traverse the next three retinal layers in a highly polarized fashion. The outer and inner segments of rods and cone-shaped photoreceptors about the RPE and form the rod/cone layer. The outer limiting membrane (OLM), comprising adherens junctions between photoreceptors and supporting Müller glia, comes next and provides structural support as well as a barrier function for the retina. The outer nuclear layer (ONL) contains photoreceptor nuclei and is located on the other side of the OLM followed by the outer plexiform layer (OPL), which contains photoreceptor synaptic bodies [10] (Fig. 62.1). There are about 120 million rods and 6 million cones in the human retina, with cones being highly concentrated in the macula, or center of the retina to provide central vision [10]. Within the macula, the fovea is a pit-like structure that contains the highest density of cones, which thereby provides the highest resolution visual acuity. Cones are responsible for vision and color discrimination in well-lit environments. Subtypes are classified by the absorption spectra of the light-sensitive opsin protein they contain: L cones respond to long (red) wavelengths of light, M cones respond to medium (green) wavelengths, and S forms respond to short (UV/violet or blue) wavelengths. Rods, on the other hand, are excluded from the macula and reside throughout the periphery of the retina. They contain rhodopsin that is extremely efficient at absorbing green to blue wavelengths of light to provide vision in dimly lit environments [11]. The outer segments of both rods and cones capture photons of light through ordered stacks of opsin-containing disks while the inner segments contain mitochondria to produce ATP needed to regulate ion channels.

Through a process known as visual phototransduction, captured light triggers the dissociation of retinal molecules from opsin proteins in rods and cones followed by ion channel closing, subsequent photoreceptor hyperpolarization, and inhibition of glutamate release from the synaptic region of the cells. In the dark, glutamate is received by bipolar and horizontal cells and inhibits their activity. In the light, the lack of glutamate relieves the inhibition of these retinal neurons, leading to their activation. Bipolar cells then amplify and transmit the electrical signal downstream to amacrine cells through interplay among various ON and OFF bipolar subtypes. Horizontal cells help fine-tune this signal and provide feedback to photoreceptors. Nuclei of all three classes of secondary neurons, bipolar, horizontal, and amacrine cells, reside within the inner nuclear layer (INL) while their processes either extend distally to the OPL (bipolar and horizontal) or proximally to the inner plexiform layer (IPL) as in the case of amacrine cells (Fig. 62.1). The IPL is where most

dendrites of amacrine cells synaptically connect with dendrites of retinal ganglion cells (RGCs), while the ganglion cell layer consists mainly of RGC cell bodies and a few amacrine cells. In response to receiving signals from amacrine cells, RGCs send action potentials through their axons, which comprise the nerve fiber layer, into the optic nerve to relay the visual message to the lateral geniculate nucleus of the thalamus and then into the visual cortex in the occipital lobe of the brain. The inner limiting membrane (ILM) is the innermost layer of the retina and separates the nerve fiber layer from the fluid-like vitreous humor, the large cavity located between the lens and the back of the eye. The ILM comprises Müller glia footplates and a basement membrane containing various extracellular matrix proteins. Müller glia themselves traverse all the layers of the retina to provide structural support and functional stability to all the other retinal cell types (Fig. 62.1).

Retinal degenerative diseases cause targeted dysfunction and loss of specific cell types within the retinal layers, and this in turn disrupts the visual phototransduction cascade. For example, AMD involves dysfunction and loss of RPE cells first, followed by the loss of cone photoreceptors in the macula and leading to loss of central vision. In severe cases, loss of rods and thus peripheral vision may follow. Retinitis pigmentosa (RP) involves initial loss of rod photoreceptors, leading first to loss of peripheral and night vision, while more severe cases lead to loss of cones and central vision as well. In extreme cases of RP, secondary neurons in the INL and IPL may degenerate, become disorganized, and undergo aberrant rewiring due to loss of proper input from the ONL [12]. Glaucoma involves damage to the axons of RGCs in the proximal-most layer of the retina, often leading to their death and progressive loss of vision. Lastly, DR involves disruptions to retinal vasculature due to repeated bouts of elevated blood glucose. This disturbs blood flow and nutrient supply of neuroretinal cells, leading to retinal neovascularization, neuroretinal degeneration, and ultimately visual impairment. Preclinical and clinical data suggest that engraftment of cells, which can provide functional replacement or trophic support to the impaired cell type holds therapeutic promise to treat these otherwise intractable diseases (Table 62.1). Details on each retinal disease and tissue engineering approaches to treat them are discussed in the next sections.

Age-related macular degeneration

AMD is the third leading cause of blindness worldwide after cataracts and glaucoma and the leading cause of blindness in industrialized countries [2,13]. Its global prevalence was 170 million people in 2016 and is projected to be 200 million by 2020 and 288 million by 2040 [13,14]. In the United States alone, 3 million people are projected to have AMD by 2020 [15]. As its name suggests, aging is the biggest risk factor for AMD along with environmental factors such as smoking, diet, alcohol consumption, and sunlight exposure. Evidence also suggests that there is a genetic component to AMD. In a large-scale study of 16,144 late AMD patients and 17,832 controls, 34 loci encompassing 52 genetic variants independently showed association with late AMD cases [16]. There are several detailed clinical grading scales for AMD [17], although disease severity is generally classified as early, mid, and advanced stages based on the number and size of drusen, the lipid, and protein containing deposits that form between the RPE and the underlying Bruch's membrane. The "dry" form of AMD accounts for 80%–90% of all AMD cases where there is loss of RPE or geographic atrophy (GA) in the macula but without neovascularization. The "wet" or exudative form of AMD is characterized by neovascularization and is considered more severe than dry AMD as it often leads to blindness. As dry AMD worsens, 10%–20% of cases will progress to wet AMD. In situations where the disease leads to blindness, 80%–90% of them are in patients with wet AMD and up to 20% of blindness results from severe dry AMD with GA [18].

Strategies to prevent or delay progression of AMD include taking supplements containing zinc and the antioxidants, vitamins C and E, and beta carotene [19]. Further supplementation with the carotenoids, lutein and zeaxanthin and/or omega 3 fatty acids may also help, but there are conflicting reports as to the actual benefit of these strategies [20–22]. Antiangiogenic therapies, including the anti-VEGF drugs ranibizumab (brand name Lucentis), bevacizumab (Avastin), and pegaptanib (Macugen), have shown promise in preventing further visual acuity (VA) loss in wet AMD patients [23–25]. For atrophic AMD/GA, most therapies evaluated to date, including the complement inhibitors, eculizumab (Soliris) and lampalizumab, have not proven to be very effective [26–28]. Cell-based therapies may be an alternative for AMD patients who do not respond to other treatments mentioned earlier.

History of retinal pigment epithelium as a cellular therapy for age-related macular degeneration

Historically, the culture of RPE in vitro dates back to 1980, when Flood et al. [29] successfully maintained human RPE from adult donors in culture for several months. Shortly thereafter, the feasibility of RPE transplantation was demonstrated by injecting human RPE into owl monkey eyes [30,31]. In the late 1980s, groups began

TABLE 62.1 Major retinal degenerative diseases and cell-based therapies in development.

Retinal degenerative disease	Est. worldwide prevalence (million)	Cause(s), risk factors	Primary cell type affected	Cell therapies in clinical trials (or preclinical development)
AMD	200	• Age • Genetic risk (≥52 genetic variants at ≥34 loci) • Smoking • Diet and alcohol consumption • Sunlight exposure	RPE	• Adult 1 degree RPE • Fetal RPE • Adult 1 degree IPE • hESC-RPE cells and sheets • iPSC-RPE
RP	2.5	• Genetic mutations (>3100 mutations in >66 genes cause primary RP) • Genetic mutations (>1200 mutations in >31 genes cause RP as part of a larger syndrome)	Rod photoreceptors	• Adult 1 degree photoreceptor sheets • Fetal neuroretinal cells and sheets • Fetal retinal progenitors • (PSC-derived retinal cells in preclinical development)
Glaucoma	60–70	• Age • Genetic risk (>70 single-nucleotide polymorphisms associated with POAG form) • Ancestry (POAG higher in African, Hispanic populations while NTG form is more prevalent in Asian populations)	Retinal ganglion cells and TM cells	• CNTF-expressing, encapsulated ARPE-19 cell line • (PSC-derived RGC and TM cells in preclinical development)
Diabetic retinopathy	95	• Diabetes mellitus, both types I and II, particularly those with diabetes for >20 years	Retinal vasculature	• Adipose-derived MSCs • (PSC-derived vascular progenitors in preclinical development)

AMD, Age-related macular degeneration; *hESC*, human embryonic stem cell; *IPE*, iris pigment epithelium; *iPSC*, induced pluripotent stem cell; *MSC*, mesenchymal stem cell; *NTG*, normal tension glaucoma; *POAG*, primary open-angle glaucoma; *PSC*, pluripotent stem cell; *RP*, retinitis pigmentosa; *RPE*, retinal pigment epithelium; *TM*, trabecular meshwork; *CNTF*, ciliary neurotrophic factor.

using the Royal College of Surgeons (RCS) rat as a preclinical model for evaluating the therapeutic properties of transplanted RPE [32,33]. The RCS rat strain contains a mutation in the gene for MERTK, a receptor tyrosine kinase involved in RPE phagocytic functions. Without functional MERTK, RPE phagocytosis is disrupted and thus unable to support the natural process of engulfing and recycling of shed photoreceptor outer segments. This leads to atrophy of the RPE followed by the loss of photoreceptors. By 10–12 weeks of age, RCS rats are blind, providing an accelerated model of the retinal degeneration process observed in human AMD. Since the 1980s the RCS rat model has become one of the most well-studied and commonly used models to evaluate new therapies for AMD and has helped support the development of such therapies for clinical trials.

In the 1990s the first RPE transplantation studies in humans were performed, targeting patients suffering from severe AMD with GA [34–36]. A variety of different delivery methods were used in these early studies, with cells contained as part of an autologous pedicle graft, an allogeneic patch with attached Bruch's membrane, a patch containing fetal RPE, or as a cellular suspension of allogeneic RPE [34–36]. Collectively, these studies established early safety and feasibility of RPE transplantation but reported only modest if any effects on visual acuity. Rejection of the transplanted cells was also noted anywhere between 3 and 12 months following the treatment. Additional preclinical studies focused on ways to improve the survival of transplanted cells, including different immunosuppression regimens [37,38]. In the early 2000s the use of autologous pigmented epithelium from other areas of the eye was also explored. Iris pigment epithelium (IPE) [39–42] and RPE harvested from peripheral retina [43,44] or nasal retina [45,46] were transplanted onto the macula with limited success. IPE was found to phagocytose outer segments more slowly than RPE and also lacked essential retinoid metabolizing enzymes that RPE contain [40,42]. Advanced age, health of Bruch's membrane, and/or genetic predisposition to retinal

degeneration may also have precluded greater success with these autologous sources [47–49].

Many studies from 2000 to 2015 focused on the use of membranes or scaffolds to improve engraftment, orientation, and function of transplanted RPE. Potential scaffolding material should be biocompatible (i.e., nonimmunogenic) and porous to allow for diffusion of nutrients and oxygen into and waste products out of RPE. This material should also amenable to RPE attachment, viability, and growth and be flexible enough to ease of handling during surgery. Amniotic membrane [50–52], anterior lens capsule [53], and human ILM [54] are natural compounds reported to improve attachment and/or function of cells such as primary adult human RPE, fetal RPE, porcine RPE, primary human IPE, and the RPE cell line ARPE-19. Other substrates that have been tested for their use as RPE scaffolds include elastin-like recombinamers [55] and modified poly(hydroxybutyrate-*co*-hydroxyvalerate) thin films [56]. Extracellular matrix from corneal endothelial cells may also be used as a resurfacing agent to improve attachment of RPE onto aged or unhealthy Bruch's membrane [57,58]. It remains to be determined which substrate(s) or scaffold material is optimal for the health, survival, and function of RPE after transplantation.

Retinal pigment epithelium from pluripotent stem cells

Studies with primary RPE such as those described earlier helped pave the way for the generation and preclinical testing of RPE from pluripotent stem cell (PSC) sources. In 2004 Klimanskaya et al. were the first to demonstrate differentiation of human embryonic stem cells (hESCs) into RPE [59]. The hESC-RPE had characteristic RPE polygonal morphology, pigmentation, and expression of the RPE markers: bestrophin, CRALBP, and PEDF. The gene expression profiles and phagocytic properties of the hESC-RPE were also similar to those of primary RPE [59]. The hESC-RPE was injected into RCS rats prior to the naturally occurring loss of photoreceptor at postnatal day (P) 21–23 to determine if the transplanted cells could slow down or stop the retinal degenerative process. By P60, animals that had received hESC-RPE showed significantly better electroretinogram (ERG) response than untreated or sham-treated controls. By P100, treated animals displayed better visual acuity (as assessed by optomotor response) than untreated or sham-treated controls, and histology at P100 confirmed extensive preservation of photoreceptors in animals that had received the hESC-RPE transplant [60]. Follow-up dose–response studies demonstrated the long-term safety and function of hESC-RPE in RCS rats and ELOVL4 mice, which are a model for Stargardt's disease, the juvenile form of macular degeneration [61]. Studies in immunodeficient mice likewise supported their safe use by demonstrating lack of tumorigenicity from the transplanted cells [61]. A multitude of additional reports have since been published in the last 10 years describing the generation of RPE from hESCs [62–68] as well as human induced PSCs (iPSCs) [69–73]. Methods of differentiation and delivery (e.g., as cell suspension, sheets, or on scaffolds) vary in these studies, yet many of them provide additional evidence for therapeutic efficacy of PSC-derived RPE in preclinical models of retinal degeneration.

Clinical studies testing hESC-RPE in humans first began in 2011 with subretinal injections of cellular suspensions in patients with dry AMD and Stargardt's in Phase 1/2 trials [74]. Results showed the safety and tolerability of up to 150,000 cells in a single transplantation, with 18 patients (9 AMD and 9 Stargardt's) being treated. Fundus imaging taken at baseline and 3 and 6 months after transplantation showed a stable region of pigmentation in the area of the graft for several patients, which may or may not correspond to the transplanted cells (Fig. 62.2).

Overall, there was no evidence of rejection, hyperproliferation or tumorigenicity for up to 22 months following transplantation even after immunosuppression had been

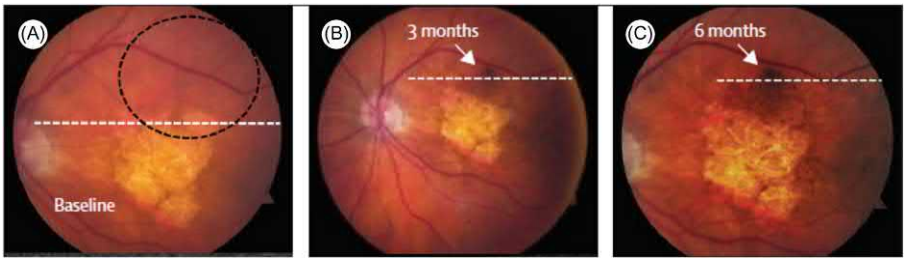

FIGURE 62.2 Fundus image of an AMD patient eye after transplantation of hESC-derived RPE shows evidence of a pigmented patch that increases with time. Dotted circle shows area for transplantation; *AMD*, Age-related macular degeneration; *hESC*, human embryonic stem cell; *RPE*, retinal pigment epithelium. *Reprinted with permission from Schwartz SD, et al. Human embryonic stem cell–derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet 2015;385(9967):509–16.*

tapered and stopped [75]. In the last 4−5 years, additional trials have confirmed the safety and tolerability of RPE cellular suspensions in patients with dry AMD or Stargardt's [76,77]. The safety and tolerability of RPE sheets in humans in AMD have now also been reported. In a Phase 1 study, a scaffold-less sheet of RPE derived from autologous iPSCs was subretinally transplanted without immunosuppression into a patient with wet AMD, following removal of the neovascular membrane [78]. A monitoring period of 12 months showed evidence of graft survival without any serious adverse events. A second patient had been planned for the study, yet concerns over the genetic stability of the autologous iPSC-RPE preparation stopped the trial short [78,79]. In another Phase 1 study, investigators transplanted a vitronectin-coated polyester membrane containing 100,000 hESC-RPE into two wet AMD patients with long-term local immunosuppression. This approach showed no evidence of adverse events in the 12 months following transplantation [80]. In a third Phase 1/2a trial, investigators transplanted a synthetic parylene substrate mimicking Bruch's membrane containing 100,000 hESC-RPE into four patients with GA dry AMD [81]. At a mean follow-up of 260 days, the graft was well tolerated with no signs of adverse events even though immunosuppression had been tapered and stopped by day 60 [81]. As these initial trials were focused on safety and often used subtherapeutic doses of RPE, follow-up clinical trials should help address the potential efficacy of PSC-RPE for preserving or improving visual acuity in patients with AMD and Stargardt's.

Retinitis pigmentosa

RP is a rare, inherited form of progressive retinal degeneration that involves development of night blindness and/or increasing loss of peripheral vision. In more severe cases, it can lead to loss of central vision and eventually blindness. It affects roughly 2.5 million people globally, or about 1 in every 4000 people [82,83]. The disease begins with the dysfunction and degeneration of rod photoreceptors in the peripheral retina followed by the loss of cones, and thus central vision. Upon clinical examination, there is evidence of pigment deposits caused by death of photoreceptors, retinal vessel attenuation, narrowing of visual field (tunnel vision), and a change in ERG patterns [83,84]. Although the age of onset, clinical features, rate of progression, and underlying genetic causes are highly variable, underlying genetic heterogeneity is a root cause. Over 3100 mutations in more than 66 genes can cause RP while more than 1200 mutations in another 31 genes cause RP as part of a larger syndrome, including Leber congenital amaurosis, Usher syndrome, Refsum disease, Bardet−Biedl syndrome, and neuropathy ataxia and RP [82,84]. RP may be inherited in an autosomal-dominant (15%−25% of cases), autosomal-recessive (5%−20%), X-linked (5%−15%), or unknown (40%−50%) manner [83]. Genes commonly associated with RP are involved in photoreceptor-specific functions such as retinal metabolism, phototransduction and outer segment structure, connecting cilium structure, rod-specific transcription factors, and splicing factors [83]. A study of 270 families with autosomal-dominant RP found that mutations in the gene encoding rhodopsin were the most common, accounting for 30.7% of cases [82]. Another commonly mutated gene in this study was RPGR, which encodes a GTPase-binding protein located in the connecting cilia, a structure that connects photoreceptor inner and outer segments. RPGR is located on the X chromosome and accounts for 70%−90% of X-linked form of RP [85].

There is no effective therapy for curing or preventing RP due to its underlying genetic cause. Supplementation with antioxidants such as vitamin A, docosahexaenoic acid, and lutein may help delay the progression of RP [86]. Other potential therapies that may delay progression include neurotrophic factors such as ciliary neurotrophic factor (CNTF) [87,88], unoprostone isopropyl, an activator of calcium-activated potassium channels [89,90], and the histone deaceylase inhibitor, valproic acid [91,92]. Yet there is still a high unmet need for therapies that can stop, prevent, or reverse vision loss in RP patients.

To be effective in treating RP, transplanted cells must engraft and either provide significant neuroprotection to save deteriorating photoreceptors or functionally compensate for photoreceptors that have already been lost. In 1987 Klassen and Lund transplanted embryonic rat retinal tissue onto the brain stem of neonatal rats, and after 5 months he found that the transplant could drive a light-stimulated pupillary reflex in the host eye. This was among the first studies to provide evidence that transplanted retinal tissue could survive and make functional synaptic connections to drive a natural response even though it was not in retina itself [93]. Isolation and transplantation of rat neonatal photoreceptors into a degenerative retinal environment was demonstrated shortly thereafter using a high-level illumination photoreceptor injury model [94]. In another study, Gouras et al. subretinally transplanted photoreceptor microaggregates or papain-dissociated photoreceptors into rd1 mice, a model in which mutation of the Pde6b gene causes early onset photoreceptor degeneration. Cells were transplanted at a time when endogenous photoreceptors have already been lost [95]. These neonatal photoreceptors survived for at least 2 months and, in the case of the transplanted microaggregates, displayed mature outer segments, which could be phagocytosed by endogenous RPE [95]. Later studies showed that wt photoreceptors could provide significant neuroprotection of cones away from the transplantation

site in rd1 mice, suggesting the secretion of neurotrophic factors could provide therapeutic effects for degenerating photoreceptors [96,97]. Additional studies were aimed at identifying the most appropriate developmental stage of photoreceptors for optimal integration, maturation, and, more importantly, greater evidence of functional synaptic connectivity to downstream retinal neurons [98–100]. Maclaren et al. showed that rod precursors isolated from postnatal day 1–7 mice, a time when the rod-specific transcription factor (NRL) is expressed, can integrate into the degenerate adult ONL and form synaptic connections, which translated into improved visual function [98]. Moreover, Bartsch et al. came to a similar conclusion that early postnatal photoreceptors represent an ideal developmental state for functional integration [99]. Additional studies provided further information regarding the visual function improvement and synaptic connectivity of transplanted photoreceptors in various models [101–103]. One such study examined the integration of NRL-GFP^{+} rod precursors in six different models of photoreceptor-focused retinal degeneration, each with its own unique features, severity, and speed of photoreceptor loss. The authors found that the amount of glial scarring and changes in the integrity of the OLM were key determinants of transplanted cell integration and visual improvement, suggesting that more than just the severity of photoreceptor loss needs to be considered when developing cell-based therapies for RP [102]. In another study, differential fluorescent labeling of donor and host cells provided evidence that transplanted photoreceptors can fuse with host cells and that cell fusion may be a previously unrecognized mechanism for the effects of transplanted cells on visual function [104]. Additional analysis suggested that cytoplasmic, but not nuclear, material passes between the cells. Differential labeling of cytoplasmic material and DNA in donor and host cells, the labeling of sex chromosomes to distinguish transplanted male photoreceptors from female recipients, and a Cre/lox-based approach (to demonstrate transfer of Cre recombinase from donor to recipient or recipient to donor) all suggested transfer of cytoplasmic material [105–107]. Cytoplasmic transfer could partially explain the visual improvement observed after photoreceptor transplantation in models when host rods and cones are still present but likely does not account for the visual improvement in models where endogenous photoreceptors are completely gone at the time of transplantation.

Photoreceptors from pluripotent stem cells

PSCs represent an attractive alternative starting material for cell-based therapies to treat RP given issues with sourcing postnatal retinal tissue in scale. In 2006 two studies demonstrated the feasibility of using hESCs as a starting material. Lamba et al. differentiated hESCs into retinal progenitors that could give rise to photoreceptor-like cells. Coculture with retinal explants, and in particular degenerative retinal explant tissue, provided appropriate cues to stimulate specific differentiation toward photoreceptors, whereas isolated retinal progenitor cultures without the explant tended to differentiate more toward ganglion and amacrine cells [108]. In the same year, Banin et al. showed that hESC-derived neural progenitors could give rise to cells expressing key photoreceptor markers upon subretinal transplantation in rats [109]. Lamba et al. confirmed that their retinal progenitors could differentiate in vivo into photoreceptors in 2009 using the Crx$^{-/-}$ model of LCA [110]. The presence of these transplanted cells restored light responsiveness to the animals. Osakada et al. were able to avoid the use of retinal tissue to further differentiate their progenitors into photoreceptors by using retinoic acid and taurine in vitro [111]. In 2009 the same group successfully applied their protocol to iPSCs [112]. In 2010 Lamba et al. also reported the successful differentiation of iPSCs into photoreceptors. Here, they drove GFP expression off a photoreceptor-specific promoter, interphotoreceptor retinoid-binding protein (IRBP), to isolate the photoreceptors from differentiating cultures. Subretinal transplantation of the GFP + cells into wt mice showed that they survived at least 3 weeks and integrated into the retinal architecture while maintaining expression of photoreceptor-specific markers such as rhodopsin, recoverin, and Otx2 [113]. Additional studies have confirmed the ability of hESCs and iPSCs to give rise to retinal/photoreceptor progenitors using similar standard 2D, growth-factor-enriched protocols, which can last up to 4–5 months [114–116]. Among them, Barnea-Cramer et al. showed that both hESC- and human (h)iPSC-derived photoreceptor progenitors can integrate into the degenerate rd1 mouse retina following subretinal injection (Fig. 62.3).

Transplanted cells survived at least 3 weeks and expressed mature photoreceptor-specific markers such as rhodopsin, recoverin, PDE6b, and cone arrestin. Moreover, animals receiving either hESC- or hiPSC-based therapy displayed improvement in visual function compared to untreated rd1 controls, as assessed by optomotor response and light–dark box vision dependent behavioral tests [116].

Recently, several groups have been exploring 3D organoid-based cultures as a way to increase the efficiency of hESCs/iPSC differentiation into photoreceptors and more faithfully recapitulate the structured environment of the developing eye. Nakano et al. first described the generation of hESC-derived optic cups in 2012 through modification of their mouse ESC organoid culture system [117]. In this hESC 3D culture system, it was

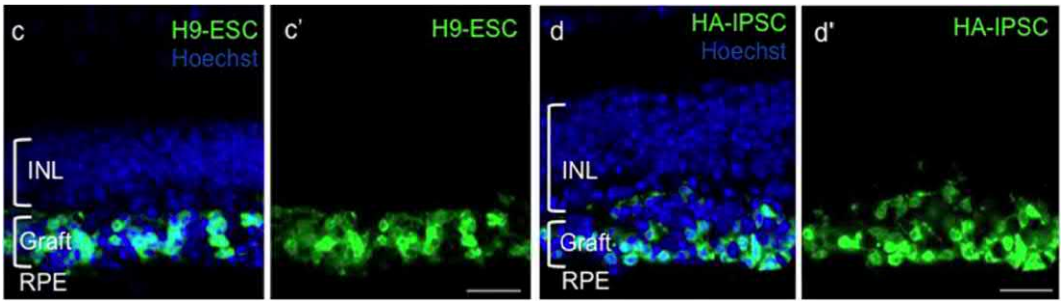

FIGURE 62.3 hESC- and iPSC-derived photoreceptor progenitors engraft and survive in rd1 mice, a model of severe photoreceptor degeneration. GFP-labeled H9 hESC-photoreceptors (left two panels) or HA-human iPSC-derived photoreceptors (right two panels) engraft between the RPE and INL of retina in rd1 mice 3 weeks after subretinal injection. Scale bar, 25 μm; *hESC*, Human embryonic stem cell; *INL*, inner nuclear layer; *iPSC*, induced pluripotent stem cell; *RPE*, retinal pigment epithelium. *Reprinted with permission from Barnea-Cramer AO, et al. Function of human pluripotent stem cell–derived photoreceptor progenitors in blind mice. Sci Rep 2016;6:29784.*

found that Notch inhibition starting at day 29 and lasting for ~2 weeks can increase the percentage of recoverin containing photoreceptors up to 78%. They have since used an improved version of their 3D protocol to generate retinal sheets for transplantation, showing engraftment of the sheets in two monkey models of retinal degeneration [118]. They have also now demonstrated survival and maturation of the retinal sheets in two immunodeficient mouse models of retinal degeneration, where retinas from animals that received the sheets showed evidence of greater light-driven responses on multielectrode array recordings than those without the transplanted sheets [119]. Additional groups have also reported the use of 3D organoid technology for human PSC differentiation into stratified retinal tissue, including photoreceptors [120–124].

The technical feasibility and safety of subretinal injections of retinal tissue and cell suspensions to treat RP were first explored in the late 1990s and continue to be explored today. In 1997 cadaveric sheets of photoreceptors were harvested and subretinally transplanted into two RP patients without immunosuppression. Although no improvements in visual function were observed, there were no signs of adverse events or rejection either [125]. In 1999 Das et al. subretinally injected a suspension of cells isolated from human fetal neuroretinal tissue (from 14 to 18 weeks gestational period) into 14 RP patients. The 40-month follow-up period suggested no major adverse effects, except for a single case of retinal detachment in one patient, which was noted 3 days after the surgery [126]. In the same year, Radtke et al. reported successful subretinal transplantation of fetal retinal tissue sheets into two patients with autosomal-recessive RP with evidence of some potential visual improvement [127]. Additional clinical studies by this same group have confirmed that transplantation of fetal retinal tissue sheets is safe and may provide visual improvement [128,129]. In one particular study, 10–15-week–old fetal neural retinal cell layers, including RPE, were transplanted into 10 patients: 6 with RP and 4 with AMD. Long-term follow-up showed that the grafts were well tolerated and potentially efficacious; 7 out of 10 patients showed some degree of sustained visual acuity improvement as assessed by EDTRS, out as far as 6 years posttransplant.

Twenty years after the first of these studies, additional groups are still testing fetal neuroretinal cells in clinical trials as a potential therapy for RP. In a 2017 study a 1×10^6 cellular suspension of 12–16-week-old fetal retinal progenitors was subretinally injected into eight patients with rod–cone dystrophy while 1×10^5 of the same cells was injected into RCS rats for comparison [130]. Six weeks postinjection, the preclinical study showed that the retinal progenitor cells (RPCs) engrafted into the host ONL and expressed photoreceptor markers, recoverin and rhodopsin. Animals given the cell therapy also showed improved ERG b-wave recording compared to nontreated animals. In the clinical study the cells were well tolerated yet visual acuity only improved transiently; improvements were seen between 2 and 6 months postsurgery but returned to pretreatment baseline levels by 12–24 months [130]. Two companies, jCyte and ReNeuron are also testing cellular suspensions of fetal retinal neuronal progenitors for RP. In December 2017 jCyte reported intravitreal injection of their fetal RPC suspension, jCell, which showed a favorable safety profile and potential therapeutic benefit in a Phase 1/2a trial involving 28 RP patients [131]. They are currently conducting a Phase 2b masked and controlled follow-up clinical trial with 85 RP patients using BCVA, visual fields, contrast sensitivity, quality of life metrics, and maze navigation ability as endpoints. ReNeuron is testing subretinal injection of their human RPCs (hRPCs) in Phase 1/2 clinical trials for RP as well. The ReNeuron hRPCs are from a 10- to 12-week-old human fetal source conditionally immortalized with a temperature-sensitive SV40 antigen [132] to allow expansion of the progenitors only at the permissive temperature of 33°C instead of at body

temperature of 37°C. Preclinical data obtained 12 weeks posttransplantation suggested subretinal injection of these progenitors preserved the ONL and visual function in RCS rats, likely through paracrine-mediated trophic support. The engrafted cells did not stain positive for any photoreceptor markers upon histologic evaluation, suggesting they did not functionally replace photoreceptors but likely rescued the function of endogenous photoreceptors [133]. ReNeuron expects to report clinical trial results in mid-2019. Retinal progenitors or photoreceptor precursors derived from human PSCs will likely also enter clinical trials as a potential therapy for RP within the next 10 years although it remains to be determined if they will be in the form of cellular suspensions or engineered onto sheets.

Glaucoma

Glaucoma involves damage to the optic nerve and degeneration of RGCs, the results of which can lead to visual impairment and, if left untreated, blindness. It affects 60−70 million people worldwide and of these, 8.4 million will go blind [134,135]. Normal aging and genetics both contribute to increased risk of developing glaucoma. Glaucoma can be classified based on clinical features of the disease. Primary open-angle glaucoma (POAG) is a leading cause of irreversible blindness globally [136] and accounts for nearly 90% of all glaucoma cases. People of African or Hispanic ancestry appear to be more at risk for POAG than those of European ancestry. Other forms of glaucoma include normal tension glaucoma (NTG), congenital glaucoma, and angle-closure glaucoma. The differences between these forms and underlying pathophysiology of glaucoma are as follows. POAG is characterized by elevated IOP (eIOP) (e.g., $\geq$21 mmHg). The ciliary body, located at the front of the eye, generates aqueous humor that flows into the anterior chamber. Aqueous humor provides sufficient ocular pressure to keep the spherical shape of eye as well as helps transport nutrients and antioxidants. Normal efflux of aqueous humor out of the anterior chamber occurs at the angle between the iris and cornea. Trabecular meshwork (TM), Schlemm's canal, collector channels, and aqueous veins residing at this angle represent the normal outflow pathways. In POAG, eIOP occurs even though this angle is open. The normal aging process causes outflow to be less efficient. Increasing age leads to a decline in TM cellularity and health. In glaucoma, accumulation of extracellular matrix and reactive oxygen species contributes to senescence and death of TM cells [137]. Dysfunction of the TM in turn contributes to a blockage in the outflow of aqueous humor and leads to the elevation in IOP. Long-term eIOP blocks retro- and anterograde transport of neurotrophins along RGC axons at the optic nerve head, which normally serve to maintain RGC survival and health [138]. Through this sequence of events, eIOP contributes to progressive damage of the optic nerve, which in the early stages, causes no visual symptoms and may go unnoticed. Visual impairment begins to occur when the progressive degeneration of the optic nerve and RGC axons hinders normal visual transduction signaling to the brain. More than 70 single-nucleotide polymorphisms have been found to be associated with POAG, including those in the genes for myocilin, optineurin, neurotrophin 4, and caveolin 1,2 [139]. POAG is usually treated with eye drops containing IOP-lowering drugs or with laser trabeculoplasty to increase outflow through the TM. Various classes of IOP drugs are used clinically and include beta blockers (e.g., betaxolol and timolol), alpha 2 adrenergic agonists (apraclonidine and brimonidine), carbonic anhydrase inhibitors (brinzolamide and dorzolamide), cholinergic muscarinic agonists (pilocarpine), and prostaglandin analogs (latanoprost and travoprost) [140]. In 2017 two new IOP-lowering drugs were approved, latanoprosten bunod, a nitric oxide-releasing prostaglandin analog, and netarsudil, a rho kinase inhibitor. Both help to loosen TM to lower IOP [140].

In NTG the angle for aqueous humor drainage is also open as it is in POAG, yet there is no eIOP and optic nerve damage occurs despite no large increase in IOP. NTG is more prevalent in Asians and usually begins at around 60 years of age. IOP-lowering drugs may be given to help slow the disease in NTG even though the IOP is not abnormally high. In angle-closure glaucoma the angle for aqueous humor drainage is partially blocked or too narrow. This usually presents in an acute situation, with severe pain, nausea, and red eyes, and can best be treated with trabeculectomy surgery, which involves making a small hole in the sclera to allow aqueous humor outflow [141]. Congenital glaucoma presents at birth and can be treated with surgery.

Vascular dysregulation, including changes in blood pressure, disruptions in ocular blood flow, endothelial dysfunction, vasoconstriction, systemic hypotension, and vascular inflammatory conditions, can be the source of pathophysiologic stress and development of glaucomatous optic neuropathy (GON). Migraine headaches, autoimmune diseases, and obstructive sleep apnea represent risk factors for developing GON. On a mechanistic level, oxidative stress and neuroinflammation from the abovementioned situations can promote demyelination of the optic nerve, axonal damage to RGC, and eventually RGC apoptosis. IOP-lowering drugs are the mainstay of currently available glaucoma treatments, yet progression of the disease may still be difficult to control. Patient compliance with administering eye drops at prescribed intervals or failure to administer the drops properly can be problematic. Moreover, IOP measurements taken at the doctor's office may underestimate elevations that occur at night.

24-hour IOP monitoring devices are in development [142], yet the search for additional therapies to help treat glaucoma is ongoing.

Stem cell–based therapies to treat glaucoma

Regenerative medicine approaches to treat glaucoma are an active area of investigation. Cell-based therapies fall into three main categories: (1) support for or replacement of TM to lower IOP, (2) neuroprotection to slow down or stop progressive degeneration of the optic nerve and RGC, and (3) replacement of RGCs already lost in progressive stages of disease. In 2006 Gonzalez et al. reported the identification of resident stem cell/progenitor population in isolated human TM cultures [143], suggesting they may be exploited as a source of therapeutic cells. A few years later, Du et al. isolated primary human TM stem cells and showed that, upon injection into the anterior chamber of wt mice, these cells could home to the TM, survive at least 4 months, and express the characteristic TM marker, CHI3L1 [144,145]. Another report describes optimization methods for the isolation and culture of these primary human TM stem cells/progenitors [146]. Investigators are now exploring the use of iPSCs to generate TM cells. Mouse iPSC–derived TM cells increased aqueous humor outflow and restored normal IOP levels upon transplantation into a transgenic mouse model of glaucoma, Tg-Myoc^{Y437H} [147,148], largely through their ability to elicit a proliferative response of endogenous TM cells. These therapeutic effects were observed when the cells were injected into both 4- and 6-month-old animals, which display moderate and severe disease phenotypes, respectively. Human iPSCs can also differentiate into TM stem cells and progenitors. Abu-Hassan et al. cultured hiPSCs on TM-extracellular matrix to promote their differentiation. They demonstrated that these iPSC-TM progenitors were able to restore IOP homeostasis in an ex vivo human organ culture system. In this system, resident TM cells were depleted from the anterior chamber to drive up IOP; transplantation of the hiPSC-TM progenitors could restore IOP homeostasis, whereas negative control cells had no effect [149].

Researchers are developing neuroprotective cell-based therapies to treat degenerating optic nerve and RGCs as well. The most advanced of these approaches is already in clinical trials and involves encapsulated ARPE-19 cells (an RPE cell line) engineered to overexpress CNTF. The encapsulation device measures 1 mm in diameter and 6 mm in length and consists of a semipermeable polyethersulfone exterior membrane and an interior matrix of polyethylene terephthalate with 200,000 embedded CNTF-secreting ARPE-19 cells [150]. This combination product, termed NT-501, has been shown to be safe in clinical trials for GA–AMD and RP and was found to provide a constant source of CNTF for at least 2 years following transplantation [150]. More recently, it has been found to be safe for use in macular telangiectasia type 2 with evidence suggesting it can slow the progression of disease [151]. Intravitreal implantation of NT-501 is being tested in a Phase 2 trial (https://clinicaltrials.gov, NCT02862938) for glaucoma as well.

Another cell-based therapy approach under development involves intravitreal transplantation of RGCs. Here, transplanted cells may provide neuroprotection and/or functional replacement of degenerating RGCs. Three different sources of RGCs are under evaluation, including primary, Müller glia derived, and PSC derived. For primary RGCs, Venugopalan et al. demonstrated that RGCs, isolated from p1-5 GFP$^+$ mouse retinas, could engraft cross-species into recipient Sprague-Dawley rats following intravitreal transplantation. The transplanted cells oriented their axons toward the optic nerve and extended neurites toward the optic nerve head. A small percentage of them integrated into the host RGC layer with many dendrites extending into the IPL. Evidence suggested that they made synaptic connections with and responded to light-induced signaling from host cells [152].

Reports also indicate that Müller glia may be a suitable starting material for the generation of RGCs. Singhal et al. isolated human Müller glia and differentiated them in vitro toward a Brn3b+ RGC phenotype. These cells were transplanted into an N-methyl-D-aspartate (NMDA)-induced RGC depletion rat model along with an antiinflammatory, triamcinolone, and matrix-degrading enzyme, chondroitinase ABC, to facilitate their engraftment. Four weeks posttransplantation, animals treated with the cell therapy showed an improvement in negative scotopic threshold response on ERG recording, a readout of RGC function, and the authors suggest the effect may be from neuroprotective effects but cannot rule out the potential for synaptic connections also contributing [153]. Another study reported similar therapeutic effects using feline Müller glia–derived RGCs. In this study, cells were seeded onto collagen scaffolds to facilitate their survival and attachment upon allogeneic transplantation into an NMDA RGC ablation feline model. Two weeks after transplantation, animals that had received the cells showed improved scotopic threshold response on ERG versus controls. The cells did not show evidence of integration with host retinal layers, therefore the authors concluded the improved visual function may be due to neuroprotection of residual RGCs, or regrowth of axons due to secretion of neurotrophic factors from the transplanted cells [154].

Several groups have been developing 2D culture protocols and/or optimizing protocols with chemically defined

media to generate RGCs from PSCs [155–160]. In another study, immunopanning of dissociated 3D organoids was used to isolate RGCs differentiated from hiPSCs [161]. In many studies, in vitro characterization of the resulting cells has confirmed expression of RGC markers such as Atoh, beta 3 tubulin, and Brn3b. In some studies, electrophysiologic characterization confirmed the ability of the RGCs to display action potentials, as well as spontaneous and evoked postsynaptic currents [156,159]. Some investigators also consider the length of neurite outgrowth from PSC-differentiated cells as being a desirable RGC-like characteristic [158,161]. One study also reported the presence of rare, melanopsin-expressing RGC cells in their differentiating cultures, potentially reflective of intrinsically photosensitive RGCs [157]. Although none of these studies report on in vivo transplantation of PSC–RGCs, two studies have reported that retinal progenitors can engraft into RGC layers upon intravitreal transplantation [162,163]. In one of these studies, investigators transplanted the progenitors into an NMDA-induced RGC depletion mouse model. Four to five weeks posttransplantation, cells were found within the RGC layer and some even expressed the RGC marker, Brn3a [163]. At this point, it is unclear which starting cell source and developmental stage of RGCs may be best for engraftment and long-term therapeutic benefit in a glaucomatous setting. It still also remains to be determined if transplanted cells will be able to functionally replace RGCs lost due to degeneration or if neuroprotection will serve as the main mechanism of therapeutic action.

Diabetic retinopathy

Hyperglycemia, or elevated blood sugar, may occur chronically in diabetes and can lead to a condition known as DR, which involves the degeneration of retinal vasculature leading to visual disturbances or even blindness. Oxidative stress, mitochondrial dysfunction, and inflammation from the chronic exposure to hyperglycemia lead to thickening of vascular basement membrane and premature death of endothelial cells, pericytes, and vascular smooth muscle that comprise the blood vessels. Degeneration of the vasculature impairs the delivery of oxygen to the retina, which may result in areas of ischemic death of retinal cells, including RGCs and photoreceptors, which may be apparent upon optical coherence tomography imaging as thinning of retinal layers. Of the estimated 285 million people worldwide who suffer from diabetes, one-third of them will likely develop some form of DR in their life [164]. DR can be classified by the stage of severity, beginning with nonproliferative DR (NPDR). Mild NPDR involves the accumulating appearance of microaneurysms and recruitment of inflammatory cells. Moderate NPDR also includes some swelling of retinal vessels, and, in severe cases, NPDR can involve many more vessels, leading to the secretion of pathologic cytokines, such as TNFα, IL-8, TGFb1, iNOS, and angiogenic factors such as VEGF [165]. Secretion of VEGF in severe NPDR can lead to proliferative DR, which is characterized by the appearance of new, pathogenic, fragile, and leaky blood vessels, often with scar tissue that may also cause retinal detachment. During the course of DR, leakage of blood vessels causes breakdown of the blood retinal barrier and may contribute to swelling of the macula, a condition termed diabetic macular edema, which can occur at any stage of DR and is a common cause of blindness [164].

Standard of care for DR includes glucocorticoids, anti-VEGF, and laser photocoagulation, yet none of these therapies can reverse or repair ischemic damage to tissues. Other therapies in development include other mediators of angiogenic signaling, immunosuppressants, NSAIDs, and oxidative stress inhibitors [166]. Cell-based therapies that involve the transplantation of vascular progenitors may help preserve and/or replace degenerating endothelial cells, and potentially also vascular pericytes and smooth muscle. Restoration of capillary blood flow can, in turn, preserve the viability of inner retinal cells, glia, and photoreceptors. Determining the right stage of progenitor differentiation for maximal therapeutic benefit is an area of ongoing investigation. Early in embryonic development, mesoderm tissue gives rise to small regions of clustered cells termed "blood islands," which will give rise to both hematopoietic and endothelial lineages. It is thought that a bipotential progenitor, termed the hemangioblast precedes this division of labor and can give rise to both hematopoietic stem cells and endothelial progenitors [167]. Human retinal vasculature is thought to develop around 14 weeks of gestation [168]. A large body of evidence suggests vascular progenitors can be found in adult bone marrow, heart, skeletal muscle, and other tissues and contribute to various types of vascular repair and maintenance [169]. Although the human body is equipped with these progenitors, chronic diabetic hyperglycemia impairs the ability of these endogenous progenitors to properly migrate in response to the chemokine SDF-1 or repair vascular endothelium. Caballero et al. found that human vascular/endothelial progenitors from healthy donors could repair injured retinal vasculature in four different DR rodent models while the same population from diabetic donors could not [170]. This has led to the exploration of suitable, allogeneic cell sources to provide vascular repair in DR.

Stem cell–based therapies to treat diabetic retinopathy

Several studies have evaluated adult tissue–derived mesenchymal stem cells (MSCs) in preclinical models of retinal vascular injury. Adipose-derived MSCs have been

shown to facilitate repair of retinal capillary damage in streptozocin-induced diabetic rats [171]. Adipose-derived MSCs were also found to be able to differentiate into a pericyte-like cell type that is capable of providing protection in an oxygen-induced retinopathy model [172]. Another study showed that intravitreal injection of adipose MSCs can increase ERG signal and decrease vascular leakage and apoptosis in streptozocin-induced diabetic rats. Here, in vitro studies also show adipose MSCs can withstand hyperglycemic stress and can form vascular networks when cocultured with retinal endothelial cells [173]. A more recent study shows extracellular vesicles derived from adipose MSCs have therapeutic properties in a retinal ischemic reperfusion model [174]. These and other studies suggest that the main therapeutic mechanism of action for MSCs is to provide paracrine-delivered trophic support. Other cell types, which are capable of engrafting, may provide longer term therapeutic benefit.

In 2007 multiple groups reported the differentiation of hESCs into progenitors with vascular repair abilities [175–177]. For example, Lu et al. differentiated hESCs into a putative hemangioblast population, which could differentiate into both hematopoietic and endothelial lineages. Intravitreal or systemic delivery of these cells into an ischemia–reperfusion retinal injury model showed evidence of vascular repair. In addition, intravitreal injection of the cells into diabetic rats with an NPDR phenotype showed reparative potential of these cells as evidenced by their incorporation into damaged vessels (Fig. 62.4) [175].

Follow-up studies confirmed the retinal vascular-repairing properties of these hemangioblasts in an oxygen-induced retinopathy model [178]. Additional groups have confirmed that hESCs as well as iPSCs can differentiate into VPs endowed with the ability to repair ischemic retinal vasculature [179–181]. A study by Park et al. compared the reparative properties of cells derived from different iPSC lines. They showed that iPSCs derived from cord blood (CB) gave rise to CD31 + CD146 + vascular progenitors that could home and engraft into injured retinal capillaries in a retinal ischemia–reperfusion injury model out to 45 days [168]. These CB-iPSCs were a better starting an iPSC population than fibroblast-derived iPSCs, suggesting their CB origin facilitated the generation of therapeutic VPs [168]. It remains to be determined which specific vascular progenitor population may provide the best therapeutic effects for human DR. Neither hESC- nor iPSC-derived VPs have yet been tested in clinical trials, but current efforts are underway to make this a reality.

Future directions and competing therapies

As described in this chapter, several different types of cell-based regenerative medicine therapies are under development to treat retinal degenerative diseases that arise due to aging, diabetes, and/or inherited mutations. Among these, RPE-based therapies to treat AMD are currently in clinical trials, while photoreceptor, retinal ganglion, and vascular progenitor–based therapies to treat RP, glaucoma, and DR, respectively, may enter into clinical trials within the next several years. Efforts are already under way to endow these cells with even greater therapeutic properties. Overexpression of trophic factors such

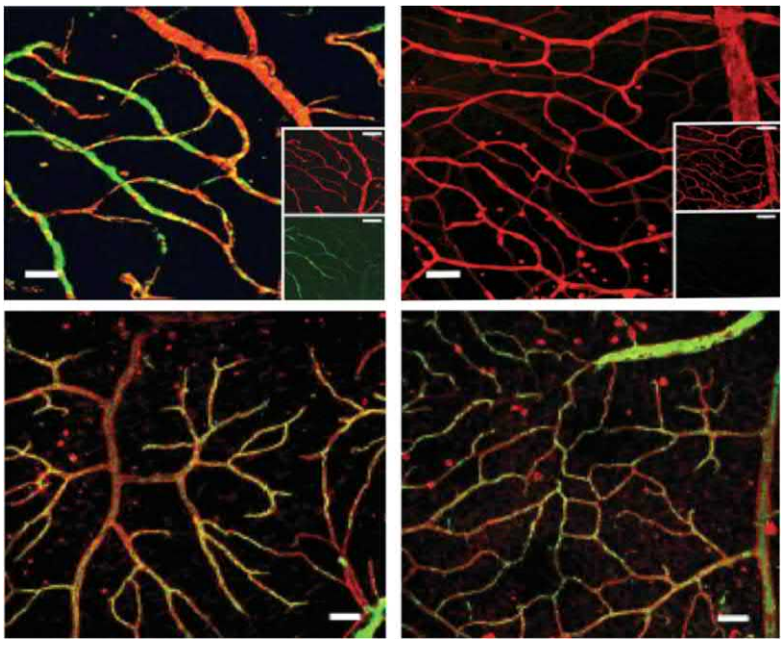

FIGURE 62.4 hESC-derived hemangioblasts incorporate into retinal vasculature of animals with retinal ischemia–reperfusion injury or nonproliferative diabetic retinopathy. *Upper panel*: (Left) Intravitreally injected, GFP (green) labeled hemangioblasts incorporate into and repair damaged vasculature in ischemia–reperfusion injured mouse eye, 1 day after injection. (Right) Retinal vasculature in normal, uninjured eye 1 day after saline injection for comparison. *Lower panel*: Intravitreally injected, GFP (green) labeled hemangioblasts incorporate into and help repair both small and large retinal vessels; images of retinal vasculature from two separate BBZDR diabetic rats are shown in left and right panels. Scale bars, 100 μm; *hESC*, Human embryonic stem cell. *Reprinted with permission from Lu SJ, et al. Generation of functional hemangioblasts from human embryonic stem cells. Nat Methods 2007;4(6):501–9.*

as brain-derived neurotrophic factor (BDNF) and CNTF may help stabilize endogenous retinal cells at risk of degeneration. Optogenetic sensors may allow the transplanted cells to overcome the loss of native photosensitive cell types [182,183]. Yet engineered cell therapy efforts will meet competition from the gene therapy field, which can deliver such factors directly to endogenous retinal cells. For example, adeno-associated virus (AAV)-mediated delivery of BDNF is being developed as a way to treat glaucoma [184]. In addition, two active Phase 1 clinical trials (NCT03326226, NCT02556736) are testing intravitreal delivery of AAV virus to deliver optogenetic sensors directly to endogenous inner retinal cells. Preclinical studies have shown that this approach can bypass degenerate photoreceptors and overcome loss of visual transduction by conferring light sensitivity to inner retinal cells instead [185–187]. Given the wide range and complex pathophysiology of retinal degenerative diseases, there is likely to be room for both approaches in the future armamentarium of treatment options.

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Chapter 63

Vision enhancement systems

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Introduction

This chapter provides an overview of visual system properties in health and disease, with an emphasis on the stages in visual-signal processing that are most commonly affected by disease and dysfunction. It then provides an overview of current approaches to vision restoration and of approaches that promise to migrate from the lab to the clinic in the coming years. In the third section the potential roles for engineered cells and tissues in this restoration process are discussed. The chapter closes with an estimate of the developments that can be expected to occur in the next 5–10 years.

Thirty years ago, the best hope vision researchers had for the enhancement of impaired vision was to build a better magnifier, by integrating optics and electronic image processing. Since then, restoration of vision to functionally blind individuals has become not just an engineering target but a realistic goal for which engineering milestones are being set and reached. The role of tissue engineering in this field may as yet be modest, but it has a great promise, as will become clear in the following pages.

In this chapter's overview of approaches to the restoration and enhancement of impaired vision in human patients, inspired by tissue-engineering principles and related technologies, we argue that the nature of eye disease and the fragility and complexity of ocular tissues such as the neural retina do not (yet) lend themselves to application of the techniques emerging in the repair of other tissues. Thus while some of the approaches presented here can function independently of tissue engineering as presented elsewhere in this volume, they at least complement those engineering approaches and lend themselves to future integration. This is particularly true in the areas of cell transplantation and neural prosthesis development, presented in the later part of this chapter, but to a lesser extent also for the optical and optoelectronic aids presented.

Visual system, architecture, and (dys) function

Human vision is mediated by one of the most highly developed sensory systems found anywhere in nature. Its capacity to combine high spatial resolution near the center of fixation with a wide peripheral field of view, accurate depth perception, color discrimination, and light–dark adaptation over 12 orders of magnitude is unparalleled. Every stage in the system is organized to accomplish this. The photoreceptor layer in the retina provides the high signal amplification of the rods required for night vision and the dense packing of three cone types required for detailed central and color vision. The intricate local preprocessing performed by subsequent retinal cell layers augments these functions by performing brightness and color comparisons and helps to condense the information stream acquired by over 100 million photoreceptors, to allow transport across a mere 1 million fibers in the optic nerve to the visual centers in the brain, where further parsing and interpretation of the image take place.

From a functional point of view, the visual system can be understood as depicted in Fig. 63.1, with sensors, preamplifiers, preprocessors, transmission lines, and several central processor stages. The two most crucial stages in this process—the conversion from light into chemical and electrical signals, and the signal transmission from the eyes toward the brain—are also the most vulnerable ones. Light conversion and signal amplification in the photoreceptors require a highly complex interplay between the molecules inside these cells and ion channels and other permeable structures in the cell membrane. These components participate in interlocking cycles of conversion and regeneration and are assisted by surrounding cells—in particular, the retinal pigmented epithelium (RPE) cells, which provide nutrients to, and digest cell membrane discarded from, the photoreceptors. Any step in this intricate

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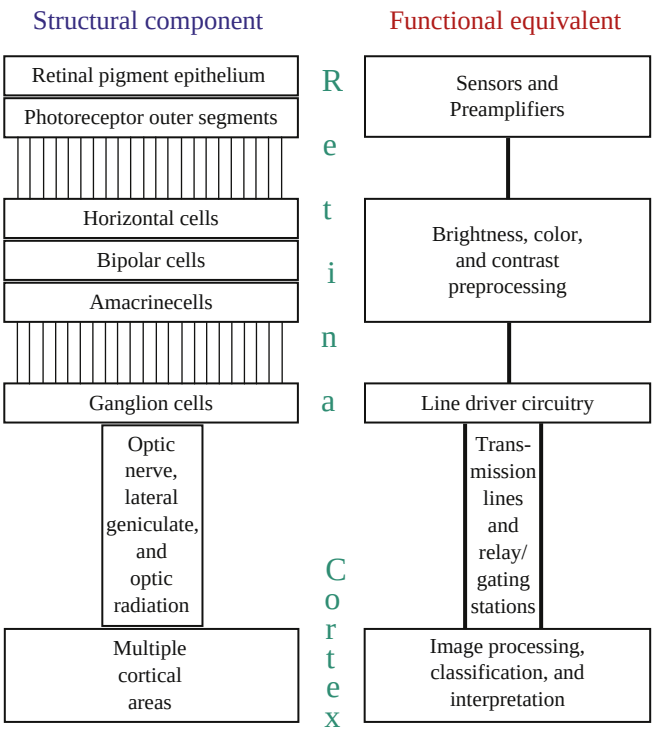

FIGURE 63.1 Schematic representation of the visual system: the outer retina (RPE and photoreceptor layers) forms the sensor array, followed by several inner retinal preprocessing stages, the ganglion cell transmission stages, and further central processing stages in subcortical and cortical brain centers. *RPE*, Retinal pigmented epithelium.

process can easily be disrupted by nutritional deficits, overexposure to short-wavelength light (presumably causing oxidative changes), attacks by pathogens, and especially genetic miscoding of one or more participating molecules. Since the mid-1980s the list of mapped (>225) and identified (>185) gene loci has grown at an ever-increasing rate. For each identified gene, there may be multiple mutations, often leading to distinct disease phenotypes; for an up-to-date list of the known mutations leading to loss of outer retinal function, see the Retnet website [1]. Not only do such mutations directly cause impaired signal transduction, but the additional energy demand, the presence of abnormal molecules, and excess shedded cell membrane may exceed the RPE cells' support capacity, which inexorably leads to degeneration of both photoreceptors and RPE cells. The most common group of disorders caused by genetic miscoding of molecules involved in the phototransduction cycle is jointly known as retinitis pigmentosa (RP), while another group is caused by a breakdown of RPE function in the central retina due to either a genetic defect (Stargardt macular dystrophy) or a combination of genetic predisposition and environmental factors [age-related macular degeneration (AMD)]. Jointly, all these disorders are known as retinal degenerations (Fig. 63.2A and B).

Retinal neurons communicate in a variety of ways, with ion-gating channels fulfilling a role in reaching and maintaining an operating level (also known as the adaptation state) and neurotransmitters and gap junctions fulfilling the principal messenger roles in carrying information in chemical and electrical form, respectively. The loss of outer retinal function, and therefore of neurochemical and electrical signal transmission to the inner retina, will affect secondary retinal cells (horizontal, bipolar, amacrine, and ganglion cells) but not necessarily threaten their survival. Until a few years ago, morphometric studies of donor retinal tissue such as that shown in Fig. 63.2B suggested survival of bipolar and ganglion cells at rates of 80% and 30% in the macula [2] and 40% and 20% at eccentricities up to 25 degrees [3], respectively, in retinae practically devoid of photoreceptors. In recent years a series of highly detailed and elegant microanatomical studies using a technique called computational molecular phenotyping [4] has demonstrated that the connectivity patterns of these inner retinal cell populations are fundamentally altered by the degeneration process (Fig. 63.3). Survival of these cells is predicated on their continued activity, and this they accomplish through the formation of new axonal and dendritic branches and through self-organization into clusters called microneuromas. These new and functionally random connections allow spontaneous oscillations to occur in the absence of meaningful input signals from the erstwhile photoreceptors, and RP patients may experience such spontaneous activities as photopsias, or "light shows" [5].

At the retinal output signal transmission stage, where retinal ganglion cells (RGCs) convert the neurotransmitter signals into electrical spike trains that travel along RGC axons toward the thalamic relay nuclei and other brain

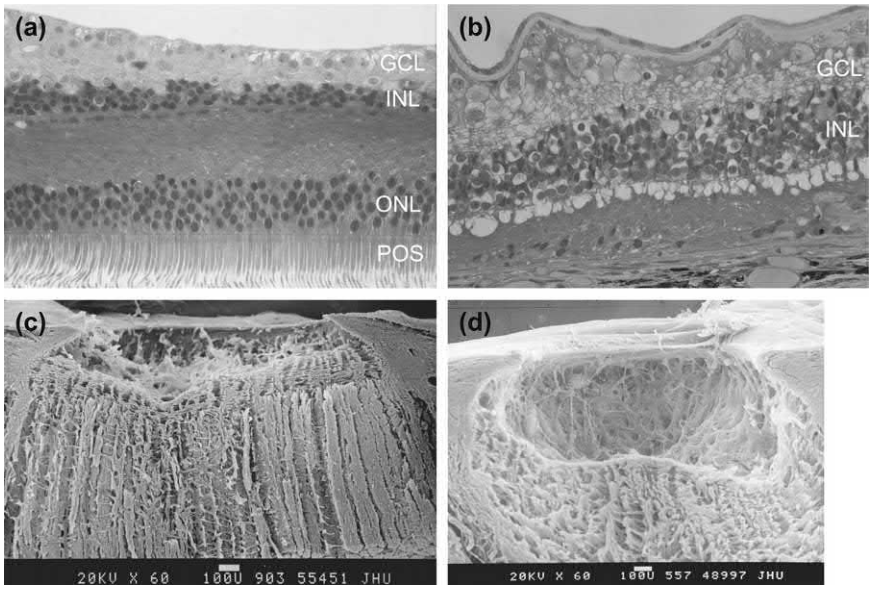

FIGURE 63.2 Representative samples of ocular morphology in healthy and diseased conditions. (A) Cross section through the retina near its center (fovea), showing healthy POS, multiple layers of photoreceptor cell nuclei in the ONL (labeled black), bipolar cell nuclei in the INL, and ganglion cell bodies in the GCL. (B) Retina of a patient with a long history of retinal degeneration and bare light perception in the last years of life, showing a lack of POS and cell bodies in comparison with (A). (C) Scanning electron microscope cross section of the optic nerve head, showing healthy appearance of the support structure, the lamina cribrosa. (D) Optic nerve head from a patient with long history of glaucoma, showing compression of the lamina cribrosa and embedded nerve fibers (RGC axons). *GCL*, Ganglion cell layer; *INL*, inner nuclear layer; *ONL*, outer nuclear layer; *POS*, photoreceptor outer segments; *RGC*, retinal ganglion cell. *(C and D) Courtesy Harry A. Quigley, M.D., the Johns Hopkins Univ., Baltimore, MD.*

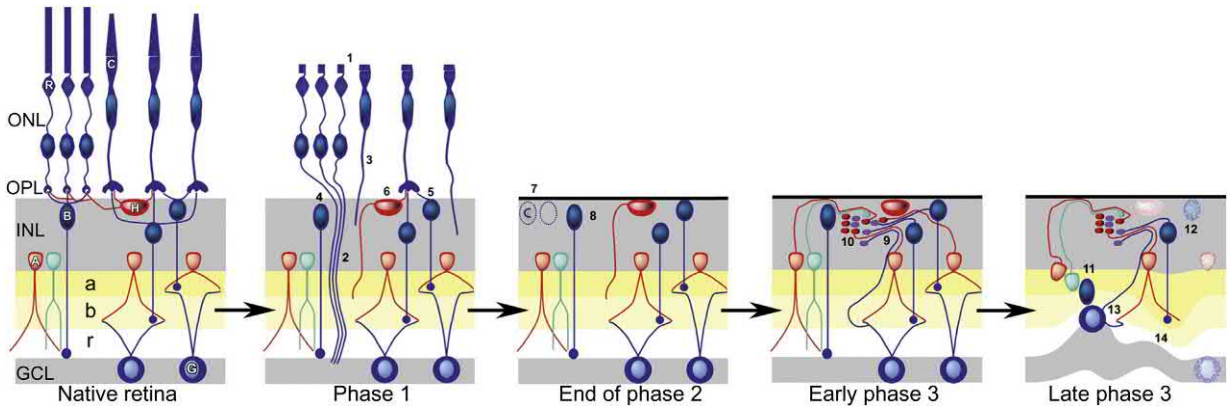

FIGURE 63.3 Simplified schematic of possible alterations during retinal remodeling. The first panel shows a representative "native" mammalian retina with its basic complement of rods (R) and cones (C) driving bipolar (B) and horizontal (H) cells. In turn, bipolar cells drive amacrine (A) and ganglion cells (G) of the proximal retina to form the major cone OFF (sublayer a), cone ON (sublayer b), and rod ON (sublayer r) zones of the IPL. Remodeling occurs in phases, as indicated under each panel. In phase 1, rod and cone stress lead to truncation of outer segments (1) and, in some instances, extension of rod (2) and cone (3) axons deep into the INL, IPL, and even GCL. Both rod and cone bipolar cells truncate their dendrites (4 and 5), and some rod bipolar cells may transiently switch to surviving cones (not shown). Horizontal cells also send axons into the IPL (6). Phase 2 is a complex period of cell death, ablation of the ONL, and resolution of the distal margin of the neural retina into a largely confluent glial seal (7) formed by the distal processes of Müller cells. Surviving neurons may continue to alter their phenotypes by changing receptor expression patterns (8), and some cone cells may even escape cell death (C). Neural remodeling becomes even more extensive during phase 3, with the formation of complex axon fascicles (9) and new synaptic complexes termed microneuromas (10). As remodeling continues throughout life, some neurons begin to migrate along glial columns (11), others die (12), and the IPL becomes transformed through new synapse formation (13) and laminar deformation (14). *GCL*, Ganglion cell layer; *IPL*, Inner plexiform layer; *ONL*, outer nuclear layer. *©2005 Robert E. Marc. Used by permission.*

areas, the system is vulnerable to mechanical insults rather than to genetic dysfunction: injury to the optic nerve (trauma), increased pressure inside the eye [crushing the fragile axon fibers at the optic nerve head (glaucoma)], and inflammation (optic neuritis) or impaired blood supply (ischemic neuropathy) of the optic nerve itself. Each of these can impair or interrupt the signal-carrying capacity of optic nerve fibers (Fig. 63.2C/D).

In addition to damage occurring at these distinct stages, more generalized damage to the retina can occur. Common mechanisms for this are the leakage of capillaries in diabetic retinopathy and the interruption of the blood supply to the inner retina (retinal vascular occlusive disease), especially in individuals with a predisposition toward the development of blood clots. Each of these can lead to widespread cell death in the inner nuclear and ganglion cell layers throughout the affected area. Vascular occlusive disease provokes angiogenesis: new retinal vessels sprout in response to growth factors signaling ischemia, but these vessels tend to leak and damage the retina's fragile structure. Diabetic retinopathy is a major cause of preventable blindness in the developed world and increasingly in developing countries as well.

Current- and near-term approaches to vision restoration

As in all biomedical engineering, approaches to restore function can be based on interruption of the disease process and tissue regeneration (autologous or grafted) or on hybrid techniques combining biological tissue with synthetic materials and devices. To support the cells affected by disease or injury, neuroprotective substances, growth factors, and genetic modifications of cell function may be used, postponing or preventing further loss of function. In addition, as long as usable function remains, one can strengthen the stimulus and internal response signal, counteracting visual impairment as much as possible.

If little or no sensor function remains, one can seek to restore it in limited form through newly grown or transplanted photoreceptors and/or RPE cells, through a prosthetic device that will electrically stimulate the remaining secondary retinal cells or through man-made tissues that mimic photoreceptor function. At the RGC level, substitution for lost signal transmission may be sought through protection of any remaining cells and administration of factors promoting axon regeneration. This may require the use of synthetic tissues and factors enabling reconnection of axons with central structures—or through in situ growth of new cells, promoted to differentiate into RGCs and to send new axons through the optic nerve.

In preliminary form, many of these approaches exist in the laboratory or are entering clinical testing, while others are merely ideas. In the following sections, both existing and prospective methods are presented.

Enhancing the stimulus through optoelectronic and optical means

Most of these devices are based on a combination of optical and electronic image enhancement techniques. Their common principle of operation is to improve visibility of the image to the diseased retina, through magnification, contrast and/or color enhancement, and filtering or feature extraction. Early incarnations, such as the low vision enhancement system—no longer in production; see Fig. 63.4A and Ref. [6]—used optical magnification, zoom, and automatic focus and—for applications limited

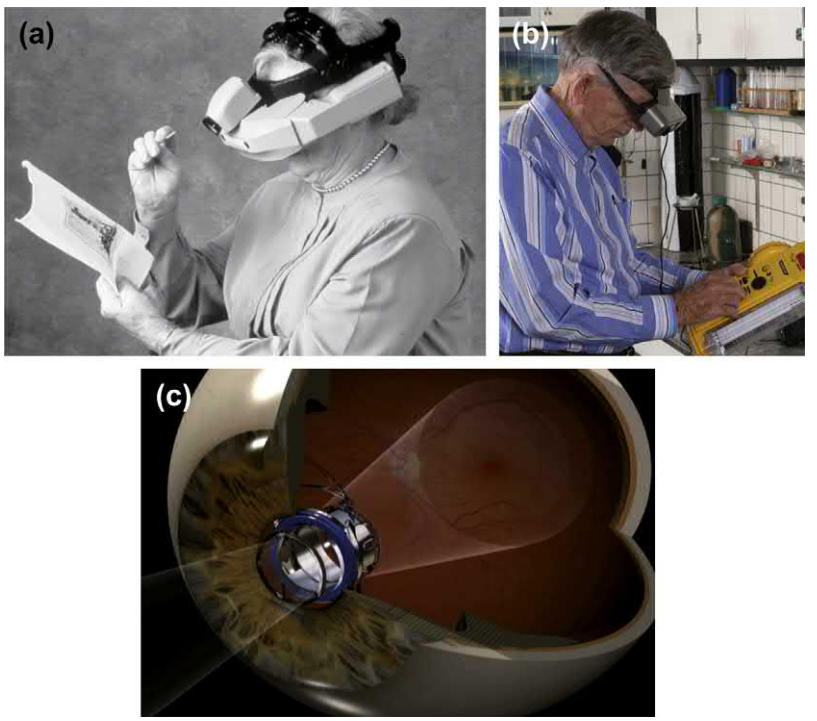

FIGURE 63.4 (A) The LVES, developed at the Johns Hopkins University's Lions Vision Center, with support from NASA and the Department of Veterans Affairs, was the first head-worn optoelectronic vision enhancement systems. This system featured binocular orientation cameras, a centrally placed 1.5−10× zoom camera with automatic focus, contrast enhancement, and a binocular projection path with 36 × 48 degrees field of view and built-in refractive correction and alignment for the wearer. (B) The eSight 3 video visor has a narrower field of view, but much higher resolution and a wide range of image enhancement capabilities. It is much lighter thanks to its LCD screens and has a full color display. (C) The Implantable Miniature Telescope (IMT provides monocular 3× magnification, to benefit patients with large central scotomas. *LVES*, Low vision enhancement system. *(B) Photo courtesy eSight Corporation and (C) diagram courtesy VisionCare Ophthalmic Technologies.*

by available contrast, such as face recognition—analog image enhancement. Digital video technology has completely replaced the analog approach, and component miniaturization and mass production have drastically reduced the production cost of head-worn video magnifiers, but today's crop of lightweight and cosmetically attractive video visors, such as Jordy (Fig. 63.4B; Enhanced Vision Systems, Huntington Beach, CA) and eSight 3 (eSight Corp, Toronto, ON, Canada), is based on the same image enhancement principles. More advanced image processing techniques such as gaze-contingent remapping, currently under development across the video gaming and virtual reality market, will allow wearers to avoid losing crucial information in blind areas of the visual field. Field of view and angular resolution, that is, the number of pixels across the screen, have also dramatically improved in the past decades. The widespread penetration of high-definition video and telepresence applications in industry and entertainment is enabling cost-effective application of this technology to low vision aids. Similarly, smaller, lighter, and more luminous flat panel displays are allowing these devices to become light and cosmetically acceptable.

A useful property of the cameras in portable video visors is their built-in automatic gain control, resulting in constant internal image brightness over a wide range of environmental illumination levels. This makes these image acquisition systems highly suitable for use in prosthetic and tissue-based image enhancement systems.

Optical systems can also be used to increase the visibility of the stimulus. One such system, approved by the US Food and Drug Administration (USFDA) for clinical application in 2011 and aimed at patients with a large central scotoma (blind spot) in both eyes due to advanced AMD, is an implantable miniature telescope (IMT, Fig. 63.4C; VisionCare Ophthalmic Technologies, Saratoga, CA), placed behind the iris in the location of the crystalline lens. It provides one eye with a magnified view of the central visual field, while the other eye is not implanted, to preserve the wide peripheral view of the fellow eye.

Following an adaptation and rehabilitation training period of 3–6 months, most wearers have no difficulty switching between the magnified view in the implanted eye and the unenhanced view in the fellow eye. Note that this is a true prosthetic device, since it is permanent.

A different type of optical vision restoration is used in patients with recurrent corneal opacification and failed corneal transplants. This so-called osteo-odonto-keratoprosthesis was first developed by Strampelli et al. [7] and typically uses a slice of autologous tooth or bone, with a small lens in the center, to replace the central portion of the opaque cornea, this restoring an image to the retina. More modern synthetic devices, such as the Boston type 1 keratoprosthesis (KPro; J.G. Machinw Co Inc, Woburn, MA), have not been compared in randomized trials against the original device, but the general impression is that the results are similar. Failure rates of the KPro are on the order of 10% after 2 years [8], suggesting that there is a rich opportunity for tissue engineers to make further progress in this area.

Visual prostheses based on electrical tissue stimulation

As was already mentioned, retinae with severe degeneration of the sensor layer retain high numbers of secondary cells. In analogy with the principle of operation of the cochlear prosthesis, that is, restoring limited hearing through stimulation of spiral ganglion cells in the cochlea [9], this provides the opportunity to convey rudimentary vision to the degenerated retina by a prosthetic device stimulating remaining secondary cells with a two-dimensional array of microelectrodes, reminiscent of the images produced by dot matrix printers a few decades ago [10]. From an engineering point of view, one can envisage a range of possible approaches (stimulating electrodes under vs over the retina; fully integrated photosensing, image processing, and stimulating systems vs external image capture and processing linked to an intraocular stimulating matrix, to name a few) but also a host of questions concerning biocompatibility, signal processing, and power management.

The most pressing question, conveying, whether visual imagery can be conveyed to patients blind from retinal degeneration, has been answered affirmatively. In a series of experiments started at Duke University in 1992, continued from 1993 until 2000 at the Johns Hopkins Wilmer Eye Institute, and since then confirmed through similar experiments in university clinics around the world, volunteers with end-stage RP—whose remaining vision was limited to, at best, light perception—as well as several patients with advanced AMD have participated in tests where, during a surgical procedure under local anesthesia, the inner surface of the retina was electrically stimulated with small and brief biphasic current pulses applied through a single or multiple electrodes [11–13]. Among the most salient findings are the subjects' ability to see small punctate light flashes (phosphenes), whose perceived location corresponds exactly to that of the stimulation, and the ability to see simple patterns of multiple phosphenes when multiple electrodes are activated simultaneously. Stimulation at rates greater than 40–60 pulses/s is perceived as continuous stimulation, and perceived stimulus intensity increases with pulse duration and amplitude as well as repetition rate. Independent tests in blind volunteers and in amphibian retina [14] have demonstrated that stimulus pulses 1 ms or longer in duration preferentially stimulate the (deeper) bipolar cells rather than the (more superficial) RGCs.

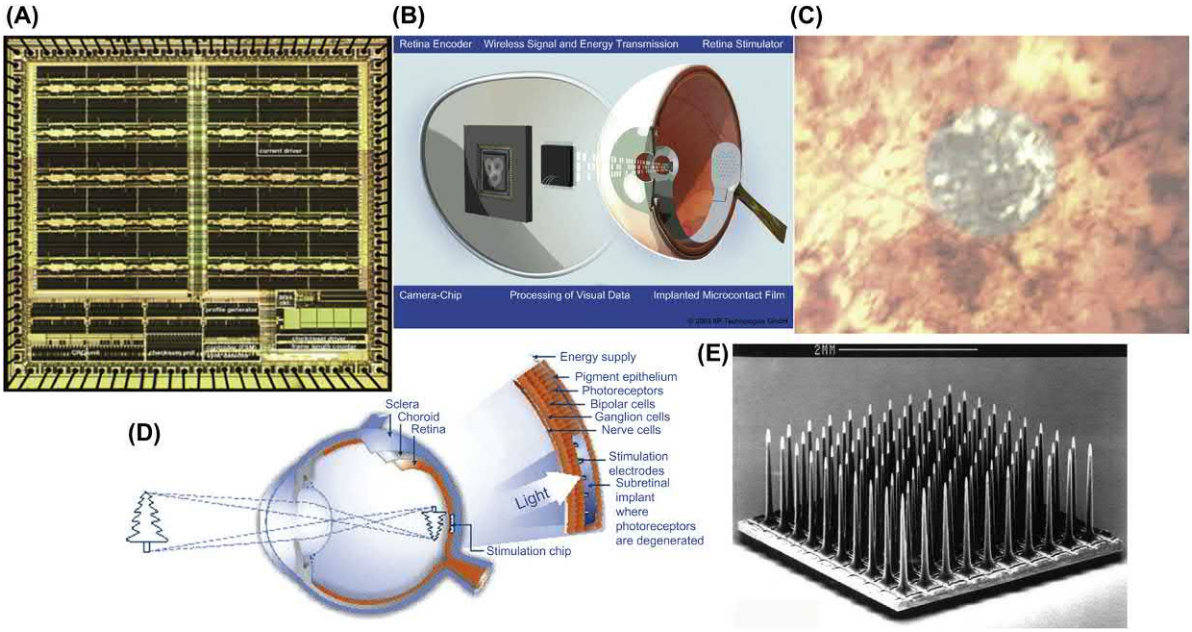

FIGURE 63.5 (A) Photomicrograph of Die3 chip, developed at the University of California Santa Cruz for reception, decoding and driving of 60 intraocular multielectrode signals. Each driver unit contains circuitry to drive five electrodes. (B) Schematic epiretinal implant with transmission of electrode control signals (IR) and energy (RF) to the intraocular decoder chip. (C) Optobionics Artificial Silicon Retina (ASR) chip in situ under the retina of a late-stage RP patient. (D) Schematic subretinal implant with external energy supply and on-chip image capture and signal amplification and conditioning. (E) The WFMA developed at the IIT as a 16-electrode module for intracortical stimulation. Multiple units are to be placed under the dura, with electrode tips reaching cortical layer IVc. *IIT*, Illinois Institute of Technology; *RP*, retinitis pigmentosa; *WFMA*, wireless floating multielectrode array. *(A) Photo courtesy Wentai Liu, Ph.D., UCSC, Santa Cruz, CA; (B) Diagram courtesy Pixium Vision SA; (C) Photo courtesy Optobionics Corporation; (D) Diagram courtesy Retina Implant AG; and (E) Image courtesy Philip Troyk, Ph.D., IIT.*

Over the past 15 years, research in this field has moved from acute experiments to chronic implants, and academic research has been joined by start-up companies and in some cases government labs to develop dedicated technologies enabling chronic implants. A good example is the relocation of the implant group formerly at Johns Hopkins to the Doheny Retina Institute at the University of Southern California, the founding of Second Sight Medical Products LLC (SSMP; Sylmar, CA) as a corporate partner, and the collaboration of these two entities with laboratories at state universities [e.g., the University of California in Santa Cruz (Fig. 63.5A), San Diego, and Berkeley] and in the US Department of Energy (Oak Ridge, Sandia, Livermore). The SSMP-led consortium, with cumulative private and public funding levels of $100 M each, introduced the 16-electrode Argus 16 to clinical testing in 2003 and the 60-electrode Argus 2 in 2007. Following a clinical trial with 30 end-stage RP patients in the United States and Europe, the Argus 2 was approved for routine clinical implantation in Europe in 2011, with a more limited approval in North America, under a humanitarian device exemption by the USFDA, granted in early 2013. Recipients of the device during the clinical trial have demonstrated the ability to localize and track objects [15,16] and recognize high-contrast letters and short words [17].

Simultaneously, several other groups are pursuing intraocular prosthetic devices. Such devices come in two variants. Stimulating "photodiode arrays," typically placed under the retina (subretinally) and stimulating electrode arrays with external image capture and preprocessing placed between sclera and choroid, subretinally or epiretinally. The latter, more common, prosthesis type is being pursued by additional groups in the United States—Harvard/MIT [12], France—Pixium (Fig. 63.5B), and a consortium of German universities [18]. All three groups have designed prototypes with 50 or more electrodes and wireless transmission to the freely moving eye, but only Pixium has been able to test such prototypes in a series of clinical pilot studies [19] and has obtained CE mark approval for clinical use in Europe. Other groups, most notably one in Korea, are experimenting with similar implant designs [20]. Other groups seek to stimulate the retina from outside the eyeball, to avoid the risk associated with retinal surgery, while accepting the reduced resolution inherent in the greater separation between electrodes and target cells.

The less common type of retinal prosthesis, a subretinal chip that uses photovoltaic elements for image capture in the location once occupied by the native photoreceptors, is conceptually simpler but in practice more challenging. In a primitive version [21] (Fig. 63.5C)

of the subretinal array, a 2-mm diameter silicon chip with approximately 5000 small photodiode units was placed under the retina in 20 late stage RP patients with remaining central vision. These devices were located near the arcades, well away from the area of functional photoreceptors. The photodiodes converted incident light into small DC electrical currents and, while the precise mechanism of action of these currents remains to be elucidated, it appears that they may have caused secondary release of neurotrophic factors in the surrounding retina, which in turn exerted a beneficial effect on the remaining photoreceptors. This implant may therefore have preserved some remaining vision, but it did not substitute an image by stimulating the secondary neurons, as a prosthetic implant would.

A true prosthetic, photosensitive, subretinal implant, with additional circuitry to provide signal amplification and pulse generation, was introduced in 2011 and received CE mark in 2013 (Alpha IMS; Retina Implant AG, Reutlingen, Germany) [22] (Fig. 63.5D). It is placed at the level of the missing photoreceptor/RPE layer, collects a sharp image of the outside world mediated by the eye's optics, and generates localized electrical impulses that stimulate nearby (bipolar) cells in the overlying retina. Such an integrated prosthesis has great simplicity and elegance, but its feasibility hinges on four important premises: that nutrients and oxygen from the inner retinal vasculature will suffice to nourish the retina overlying the implant; that external energy can be provided to drive the amplifier and pulse generation stages; that the heat generated by the implant's electronics can be safely dissipated into the underlying choroid without appreciably heating the overlying retina; and that the encapsulation be able to transmit light to the photosensitive elements, yet withstand the intraocular saline milieu. Power and control signals for the system electronics are supplied by a subcutaneous cable and a transdermal wireless link placed on the back of the head developers designed surgical methods to safely insert the array under the retina [23]. Clinical implantation for periods of up to a year has been carried out in several dozen patients, with positive functional outcomes reported [24], but problems with the insulating material limited its life span; the Alpha AMS, a version with improved insulation properties, developed in 2013, overcame this limitation, but due to limited commercial success the manufacturer ceased operations in 2019.

A novel, integrated, subretinal prosthesis, developed by a consortium centered at Stanford University, circumvents the need for electronic amplification, and thus an external power supply, by capturing an image of the scene in a head-worn visor, converting it into high intensity pulsed infrared monochrome image, and projecting this image onto the implant through a dichroic mirror that allows the wearer to simultaneously observe the visible scene [25]. The device entered clinical trials in patients with advanced dry AMD in Paris (2018) and Pittsburgh (2019) (Pixium Vision SA, Paris); it therefore aims to "fill in" the central scotoma left by the retinal degeneration process, while unhindered peripheral vision is maintained by virtue of the dichroic mirror.

In addition to retinal stimulation, three other approaches to electrical stimulation of the visual system are being pursued. One of these is direct stimulation of optic nerve fibers, either through a set of electrodes mounted on the inside of a cuff placed around the optic nerve [26] or through an array of penetrating electrodes placed on the inside of such a cuff [27]. Implantation around the optic nerve is surgically less invasive than placement of a device inside the eye, but it has the drawback that either large numbers of fibers are stimulated simultaneously (with the simple cuff) or correspondence between electrode location and retinal origin of the axon (and therefore location of the elicited phosphene in the visual field) cannot be predicted. Thus selective stimulation of individual optic nerve fibers will require a sophisticated mapping system to establish the correspondence between visual field locations of the perceived phosphenes and individual stimulating electrodes. A second approach to an electronic visual prosthesis is through stimulation of cells in the lateral geniculate nucleus, an important relay station along the visual pathway. Precise stimulation of the *lgn* with a large bundle of electrodes is challenging; however, since placement will have to be performed stereotaxically through small openings in the skull, similar to the placement of deep brain stimulating electrodes used to reduce the effects of parkinsonism [28]. Preliminary tests in rodents have shown the feasibility of this approach [29], but barring the development of novel electrode technologies for deep brain stimulation, it is doubtful that major developments of this approach will occur in the near future.

The oldest attempts at vision restoration involved stimulation of the visual cortex through intracranial electrodes, controlled by external image acquisition and signal-conditioning circuitry. Half a century ago, Brindley and Lewin [30] took the first steps in this direction by implanting a set of electrodes over the visual cortex of a blind volunteer; the phosphenes described by this volunteer were similar to those elicited by intraocular stimulation. More recently, a team at the National Institute for Neurological Disorders and Stroke (NINDS) performed tests with over 30 electrodes penetrating the cortical surface [31], and scientists at the University of Utah experimented with implantation of denser intracortical electrode arrays in cat and monkey

visual cortex [32] (Fig. 63.5E). The same group was also the first to perform simulation studies to establish minimum requirements for prosthetic vision [33], an approach followed by several other groups (for a review, see Dagnelie [34]). The cortical work that originated at NINDS continued through primate studies at the Illinois Institute of Technology (IIT) [35] and through evaluation of unsuccessful previous approaches [36,37]. A consortium centered at IIT has developed modular 16-electrode implants that are implanted subdurally, with electrodes penetrating into the visual cortex; the implants are powered and controlled wirelessly. Human implantation, expected in the near future.

In a separate development, SSMP (Sylmar, CA) has developed a 60-electrode array (Orion) based on the Argus II retinal implant and is conducting a feasibility study of cortical surface stimulation. The array is placed subdurally over the peripheral visual field projection on the medial wall of one cortical hemisphere, thus generating phosphenes away from fixation.

The cortical prosthesis bypasses both retinal and optic nerve problems and might therefore be seen as a universal approach to vision restoration. Admittedly, it may be the only viable approach for patients whose inner retina and/or optic nerves have been destroyed by glaucoma or trauma. However, cortical stimulation requires complex surgery of an otherwise healthy brain; moreover, the convoluted layout of the visual cortex complicates mapping of objects and locations in the outside world into a pixelized image that can be understood by the prosthesis wearer. Algorithms to establish such a map have been tested in primates [35] and through simulations in sighted volunteers [38], but thus far only for small sets of (simulated) phosphenes. Finding an efficient approach to mapping hundreds of phosphenes will be a crucial aspect of cortical implant research.

A final point of consideration for all visual prostheses for which the input image is acquired outside the eye is the altered role of eye movements. In natural vision the intent of most eye movements is to bring an object of interest to the center of the retina, where it can be resolved with greater detail and receive directed attention. If a head-mounted or handheld camera is used for prosthetic image acquisition, movement of the camera rather than the eye will recenter the image, whereas movement of the eye is ineffective, unless it is captured by an eye tracker and used to shift the image accordingly. Thus a visual prosthesis with a head-mounted camera must be equipped with eye movement monitoring and processing capability, or the prosthesis wearer must learn to suppress eye movements as a means to acquire visual information and use camera (i.e., head or hand) movements instead.

Detailed reviews of visual prosthesis development and simulation studies can be found in Dagnelie [34,39–41].

Retinal cell transplantation

Cell transplantation in the neurosensory system can, in principle, restore function through two mechanisms: rescue of threatened cells (either by restoring a failing support system, or through trophic factors secreted by the transplanted cells) and replacement of degenerating cells and functional integration of the transplanted cells into the host tissue. Both mechanisms may play a role in retinal cell transplantation, and the distinction is not always obvious, as will become clear.

There are two important distinctions in the choice of transplant modality. First, there is the distinction between autologous cells—harvested from the same individual who is to receive them, typically following amplification and/or differentiation in tissue culture—and allografts—harvested from a different donor, typically unaffected and immunologically matched. Second, the developmental stage of the transplanted cells may range from pluripotent stem cells, to organ-specific undifferentiated cells, to postmitotic (e.g., photoreceptor precursor) cells. Since the range of possible approaches far exceeds the scope of this chapter, only major developments will be indicated; a recent review of stem cell approaches to retinal degeneration can be found in Tibbetts et al. [42,43].

Some important categories of retinal disease are mediated by loss of RPE function, and both transplantation of autologous pigment epithelium and allografts have shown success in rescuing photoreceptor function. In the Royal College of Surgeons rat, rescue of photoreceptor function can be accomplished by replacement of the degenerating RPE with a wide range of transplants, from RPE xenografts (harvested in a different species) [44] to autologous iris pigment epithelium [45]. In human retinal disease, macular translocation surgery in AMD has demonstrated the feasibility of rescuing threatened vision by relocating the neural retina over a relatively healthy area of RPE [46], although visual outcomes of this surgical approach have been mixed and appear to be highly dependent on the surgeon's skill and experience. Immunologically mismatched RPE allografts and xenografts may not provide viable treatments in human retinal disease: contrary to the neural retina, where immune response appears to be muted or absent, a slow inflammatory response to mismatched transplanted RPE tissue is commonly observed [47].

Such an inflammatory response is less likely to occur when the transplanted cells are grown in vitro. In the last few years, RPE replacement has shifted toward experimental procedures involving cultured RPE cells grown from pluripotent embryonic stem cells [48]. These cells,

which can be grown in the laboratory under conditions meeting the standards of good manufacturing practice, have now been awarded an investigational device exemption by the USFDA, and as of this writing 27 clinical trials are recruiting participants with AMD for RPE transplantation (http://www.clinicaltrials.gov, date accessed 18.04.19).

Retinal diseases that originate as a photoreceptor cell defect do not lend themselves to treatment with autologous cell transplantation, since the same defect is likely to plague the transplanted cells, unless these can be genetically modified; novel techniques such as CRISPR−Cas9 may indeed make this possible in the near future (NCT03872479; http://clinicaltrials.gov, accessed 18.04.19). As early as the mid-1990s, transplants with cell suspensions and organized sheets containing mature photoreceptors or photoreceptor precursors (with or without RPE) were used as allografts, with varying degrees of claimed efficacy. In a light-damaged rat model, morphological evidence of synapse formation [49] and behavioral evidence of regained function [50] were among the earliest indicators that transplanted photoreceptors may be capable of assuming visual function. More recent evidence in the same direction from other laboratories suggests that functional synapse formation is possible in some animal models, while in others a gliotic seal under the retina effectively inhibits integration of the transplanted tissue with the host retina [51]. The mechanisms responsible for such widely different results remain to be elucidated but are most likely associated with varying patterns of reorganization occurring in response to different retinal degeneration genotypes [4].

Fetal tissue has thus far proven more successful than fully developed retina or stem cells in forming synaptic connections and retinal morphology resembling that of intact retina, and it has become the tissue of choice in most transplantation attempts. This tissue also carries a lower risk of rejection by the host immune system, at least in photoreceptor grafts; indeed, rejection does not appear to occur in rat or mouse photoreceptor transplantation [52]. Admittedly, some caution in extrapolating this finding to other species is warranted, for immune reactions in rodents tend to be less severe than in humans. The use of fetal tissue for research and transplantation purposes is subject to legal and ethical concerns in a number of countries, and this is an important reason for continued efforts to develop transplantation techniques that use adult donor tissue.

Preliminary attempts at retinal cell transplantation in blind volunteers, performed primarily to demonstrate safety, have yielded mixed, and at best modest, results. Allografts of cultured RPE appear to provide protection to functional AMD through trophic factors, but in exudative AMD the graft is quickly overwhelmed by an inflammatory reaction [53]. Photoreceptor transplants appear to convey an improvement in vision in some cases [54], but whether this is mediated by graft−host synapse formation or trophic support to the few remaining photoreceptors remains a matter of debate [55].

The ciliary body in the human eye contains pluripotent stem cells that can, under appropriate conditions, be coaxed to differentiate into retinal neuron precursors [56]; more recently, progress in the creation of induced pluripotent stem (IPS) cells from skin biopsy has greatly expanded the potential of both auto- and allografts [57]. Theoretically, therefore, the potential for photoreceptor cell replacement from tissue culture does exist. At this time, however, any attempts to use IPS cells to replace degenerating photoreceptors in human patients through injection into the vitreous or subretinal space, as repeatedly claimed in the popular media, seem highly premature. Without thorough research in animal models of retinal degeneration, and until photoreceptor cells can be cultured with the same level of control as has been achieved for RPE cells, it is unlikely that such attempts will lead to effective and reliable vision restoration, or even to long-term survival of the remaining native photoreceptors.

Optic nerve protection and regeneration

RGCs behave as central nervous system neurons in their inability to recover from severe injury: RGCs whose axons are damaged by cutting or crushing the optic nerve undergo apoptosis within days following injury, although this particular mechanism of cell death may be restricted to certain classes of RGC [58]. And while the axons of peripheral (motor or sensory) neurons can regenerate following surgical reconnection of nerve fascia, CNS neurons appear to lose such plasticity once their original outgrowth during ontogenesis is completed, due to changes in both their internal makeup and their environment. Oligodendrocytes that form the protective myelin sheath around optic nerve axons and astrocytes appear to play a major role in preventing axon regeneration following injury [59]. It has been known since the 1980s, however, that this environment can be effectively modified. Cut optic nerve axons in the rat and cat will not form new neurites in their natural optic nerve environment, yet they can be made to regenerate into a peripheral nerve graft and reach the superior colliculus [60−62]. Two basic steps are thus necessary for therapeutic intervention in advanced optic nerve disease to become a reality: protection of RGCs from apoptosis following severe damage to the optic nerve, and axon regeneration, including formation of functional synaptic connections in the *lgn* and other midbrain target structures. In recent years a third aspect, functional enhancement of remaining RGCs, has been added to the basic glaucoma research repertoire [63].

Studies in the last decade have provided encouraging indications in both areas. Intravitreal injections of antiapoptotic drugs and neurotrophic factors can limit the loss of RGCs and prolong the window for application of other therapeutic modalities [62]. Most of these therapies are aimed at preserving the RGC soma so that it can sustain a partially damaged axon, but there is still a need for targeted therapies that can either repair damaged axons or stimulate new axon growth [64]. Some progress is being made in stimulating axonal growth: oncomodulin has been identified as a factor that is particularly effective in promoting growth following optic nerve crush [65]. In addition, there is increased interest among researchers in free radicals, immune response, and nerve growth factors in the retina remaining unbalanced even after the primary causes of glaucoma (such as increased intraocular pressure) have been treated [66]; these are areas of active investigation that may benefit from tissue-engineering insights and methodologies.

Drug delivery

Since 1995 our understanding of photoreceptor and RGC death has changed dramatically. While it was previously thought that these cells die because a functional part—the photoreceptor outer segment and the axon, respectively—becomes dysfunctional, both events are now widely understood to trigger cell death through apoptosis and similar mechanisms. Better understanding of these mechanisms has led to the search for pharmacological interventions that may prevent cell death.

The process of RGC degeneration following transsection or crushing of the optic nerve can be halted, at least in animal models, with the use of neuroprotective agents such as neurotrophin-4/5 (NT-4/5) [67]. It turns out, however, that this protection has only a limited duration. Most RGCs die within 2−4 weeks, even with sustained administration of NT-4/5 [68]. Various other neuroprotective strategies have been attempted in animal models, but their long-term efficacy as well as the feasibility of long-term delivery near the threatened site remains to be demonstrated [69].

In the case of photoreceptor degenerations, a variety of nerve growth factors and neuroprotective agents have been used, both in vitro and in animal studies, and several of these have shown promise [70]. What remains to be worked out for most of these substances is the optimal delivery route. Systemic administration is not an option, since many of these agents do not easily pass the blood−retina barrier and/or have unwanted systemic side effects. Administration as an eye drop is not effective, because the active substance would need to diffuse through the cornea or sclera, and most of it would be washed away in the tear film. Repeated injection into the vitreous is unattractive to the patient, and it is not clear how long the active substance would persist in the eye and reach the outer retina.

Three relatively recent delivery techniques have all been successfully applied to ocular drugs in recent years: delivery by macromolecules, slow-release implants, and encapsulated cell technology (ECT). The first of these approaches caused a revolution in the treatment of exudative AMD about 15 years ago and is now widely known as photodynamic therapy (PDT). Liposomes with an embedded antiangiogenic substance (verteporfin) are injected into the bloodstream, and irradiation of the retina with a low-energy laser beam is used to release verteporfin in the choroidal space under the retina. The resulting oxygen-free radicals attack vascular endothelial cells and stop angiogenesis [71]. Unfortunately, this endothelial-cell cytotoxicity is not an effective long-term treatment, so, following the demonstrated safety of locally administered vascular endothelial growth factor inhibitors, PDT has now been replaced as the treatment of choice by intravitreal injections of such substances (notably Avastin and Lucentis) [72], even though such injections have to be repeated, in most cases several times over a 6-month period. The longer term outlook for these substances is very good. They can be embedded in poly(lactic−glycolic) acid microspheres to create slow-release implants that could be inserted into the eye through a small incision and attached to its inside wall, or they may even be effective when placed on the outside of the eyeball [73]. The third delivery method, ECT, is elegantly exemplified by the NT-501-permeable membrane delivery system developed by Neurotech SA (Lincoln, RI). When loaded with a transgenic cell line producing ciliary neurotrophic factor (CNTF) and implanted in the vitreous cavity, it will release a flow of CNTF for many months [74]. Applications are sought in the area of photoreceptor protection in retinal degenerations; a Phase I trial in RP was completed successfully [75]. Follow-up studies in RP and AMD showed modest positive results, but a clinical trial using the more advanced NT-503 implant in patients with exudative AMD was halted because the amount of CNTF produced was insufficient to control the disease process, and most study participants required supplementary intravitreal injections for effective control [76].

In some diseases, even the simplest method of drug delivery may be effective. In retinal ischemia, just as for other vascular blockages, “clotbuster” drugs, such as tissue plasminogen activator, can be intravenously injected. If this is done soon (hours or even days) after the ischemic event, much of the damage may be prevented; experimental therapy with these drugs has had promising results [77].

Genetic interventions

Delivery issues also play a role in the introduction of new genes into the degenerating retina. Most retinal degenerations are genetic in nature, either inherited from one or

both parents or caused by a new mutation. The premise of gene therapy is that intervention at an early stage may restore normal function to the cell and prevent the degeneration process that might otherwise ensue. Several strategies are needed to combat inherited retinal degenerations.

- In recessively inherited diseases, both copies of a gene are defective, and an introduction of a third copy may be sufficient to achieve adequate production of a functional protein to replace the defective one encoded by the mutated gene. A virus is used to introduce a healthy copy of the gene. Following a number of demonstrations of successful gene transduction into RPE cells and photoreceptors in vitro and feasibility studies in rodents, a highly publicized study of vision rescue in a canine model of Leber congenital amaurosis (LCA) [78] provided proof that gene therapy for some human retinal degenerations may soon be a reality. Since then, several small trials in children with LCA have demonstrated some improvements in ambulatory vision, but limited long-term treatment effects; for a review, see Ref. [79].
- In X-linked disease, males carry only one copy of the X-chromosome, including the defective gene; here, too, introduction of a healthy copy of the gene may be enough to achieve normal function.
- In dominant disease, a single bad copy of the gene suffices to "poison" the delicate balance of the cellular machinery, or, at the very least, to prevent its proper function. To reduce or prevent this a therapeutic intervention must block a step along the transcription pathway from the defective gene to its product protein but not inhibit expression of the good copy of the gene; a gene for such a blocking agent could, in principle, permanently neutralize the defect. This technique has been used in a transgenic rat model, using ribozymes to block mRNA produced by the P23H mutant gene defect responsible for one to form of autosomal dominant RP [80]. In a rat model with a naturally occurring degeneration, a gene for ribozyme production was successfully introduced into photoreceptors and proved effective even when introduced at a time when many photoreceptors had already succumbed to the effect of the defective rhodopsin gene [81], which bodes well for therapeutic interventions in dominant RP. These forms of RP tend to preserve substantial levels of vision into middle age, and this remaining vision might thus be rescued.
- An entirely separate class of retinal degenerations, typically asymptomatic until middle age and often associated with multisystem disease, is caused by mutations of the maternally inherited mitochondrial DNA, responsible primarily for cellular energy supply. Mitochondrial DNA has multiple copies, and it remains unclear whether single or multiple copies are responsible for these disorders. Moreover, the mitochondrial DNA does not lend itself to normal viral transduction techniques, which adds a level of complexity to potential gene therapeutic approaches. Recent reports suggest that proteins may be used to deliver expression-blocking factors or new functional genes into the mitochondrial milieu [82].

Vision-related gene therapy research is concentrated around RP, Stargardt macular dystrophy, LCA, and related diseases with known inheritance patterns. Due to the multiple genes that can lead to these disorders and the enormous number of specific mutations that have already been identified, many gene therapeutic variants will be needed to address a substantial proportion of these disorders. This is an extremely active area of research; recent reviews can be found in Farrar et al. [83] and Smith et al. [84].

Still, this means that a true cure may become available for these retinal degenerations. AMD and optic nerve diseases such as glaucoma and ischemia, on the other hand, have no known genetic causes, though some genes associated with predisposition for such diseases have been identified. Genetic interventions may assist in preventing some forms of these diseases, but the prospects of other therapeutic approaches are more promising in the near term than those of gene therapy.

A recent finding that may play an increasingly important role in genetic as well as other therapeutic approaches is the potentially rescuing role of modifier alleles such as Nr2e3 on homeostasis in photoreceptor cells affected by a disease-causing mutation [85]; such modifiers may play a role in many inherited diseases, either alone or as an adjuvant to other forms of therapy.

Emerging application areas for engineered cells and tissues

Of the current approaches to vision enhancement considered earlier, strictly speaking only the use of stem and transgenic cells falls within the realm of tissue engineering. The complexity of ocular structures such as the retina and optic nerve poses daunting challenges to anyone seeking to recreate their function. While this may explain the lag in progress when compared to other organ systems, it should not keep researchers who are working to restore vision from drawing on the remarkable progress of tissue-engineering approaches. We next briefly consider four application areas, corresponding to the aforementioned three processing stages in the early visual system where severe vision loss may occur and the supporting retinal infrastructure.

Photosensitive structures

Current efforts in the areas of retinal cell transplantation and intraocular prosthesis design, while promising, are in no way guaranteed to lead to reliable—that is, long term and stable—restoration of useful (let alone high quality) vision. As was explained earlier, each of the approaches currently being explored has inherent drawbacks. Implanted electrode arrays require external image acquisition and preprocessing, which may necessitate real-time eye movement tracking and compensation. Integrated implants require signal amplification and thus external energy supply. Both implant types may be limited in resolution to ambulatory vision due to the 100−200 μm distance separating the electrodes from the target cells. As a rule of thumb, resolution in the underlying tissue will be no better than this distance. Retinal cell transplantation efforts are likely to be limited by the spotty record of transplanted cells in making functional contacts with the native inner retinal circuitry. And all three approaches are limited by microneuroma formation, destroying the functional diversity of the inner retina and limiting its resolution to 50−100 μm, that is, at best 20/200 visual acuity—legal blindness.

To achieve improved retinal prosthetics, electrodes will have to make more intimate contact with the target cells. If successful, this would improve resolution to the limit imposed by the repatterned inner retinal neuronal circuitry. One can envisage such a penetrating array as the Normann microelectrode in Fig. 63.5E, but the damage such an array might do to the delicate microvasculature of the retina is a distinct risk. As an alternative, one might envisage inserting or growing, in situ, an array of artificial "neurites." These might branch out of cells grown on the surface of implanted stimulating chips, penetrating the retina until they reach a specific target environment, for example, the inner nuclear layer (INL), where they would make synaptic contacts with the native cell population; these neurites would act as "transport tubes," releasing either electrical charge or a neurotransmitter that would activate the target cells. An alternative approach would be an electrode structure that stimulates nearby retinal cells to form new neurites, spurred on by favorable growth conditions and a coating on the implant. Such coatings promoting cell growth, formed by microcontact printing, are under investigation [86].

The distance between implant and target cells could also be reduced by enticing native retinal cells to migrate toward the electrode surface, adding a new twist to the cell migration seen in naturally occurring retinal repatterning [4]. Such approaches are not as farfetched as they may sound. Several groups are experimenting with surface modifications on semiconductor chips that will allow cells to adhere to these surfaces and be activated by neurotransmitters [87], while researchers at Stanford University have reported the tendency of retinal neurons to migrate around pillar-shaped electrodes forming an array placed under the photoreceptor layer (Fig. 63.6) [88]. Combinations of such technologies may lead to improved implant−tissue interfaces.

A fundamentally different approach to improving (sub)retinal prostheses may be the use of high-yield photoconversion systems. Such high-yield conversions are known to be performed by photosystem I (PSI), a macromolecule present in the membrane of thylakoids, which can be found inside the chloroplasts that give the green color to plant leaves and algae. Early experiments with thylakoids [89] showed that it is possible to anchor these structures onto a metal surface and use them as miniature photovoltaic elements. Lee et al. [90] also demonstrated that it is possible to chemically modify thylakoid surface membranes to create charge displacement in a specific direction. Kuritz et al. [91] demonstrated the ability of PSI to impart photosensitive polarization and Ca^{2+} ion movement to retinoblastoma cells in tissue culture, as a first example of cellular engineering that may eventually lead to artificial photoreceptors. The engineering successes of thylakoids and PSI open the opportunity to create cells that assume a dipole charge distribution or, with the help of intracellular electronics, produce a biphasic pulse between the "poles"; in a subsequent stage of development, automatic gain control could be incorporated, as a limited form of light/dark adaptation. If these cells can be made to attach to the outer retinal layers or to migrate into the INL, one could achieve microscopic local current sources with sufficient conversion efficiency to ensure vision at a broad range of (day)light levels. To achieve true night vision, an external device, such as a night vision scope, could be used.

Note that in this idealized situation, the synthetic "photoreceptors" might be small and sensitive enough to provide good vision without external image preprocessing (e.g., magnification and contrast/edge enhancement). It is to be expected that preliminary forms of such light-sensing units will be neither small nor sensitive enough to provide the dynamic range and resolution required; at this intermediate solution level, external image processing with an advanced portable low vision visor would be a necessary complement to this intraocular light conversion array.

Optogenetics

Since 2006 research into sight restoration has been exploring the prospects of conveying artificial phototransduction to the cell membrane of retinal and cortical neurons [92,93]. These optogenetic approaches, as termed by Deisseroth et al. [94], introduce DNA to the target cells

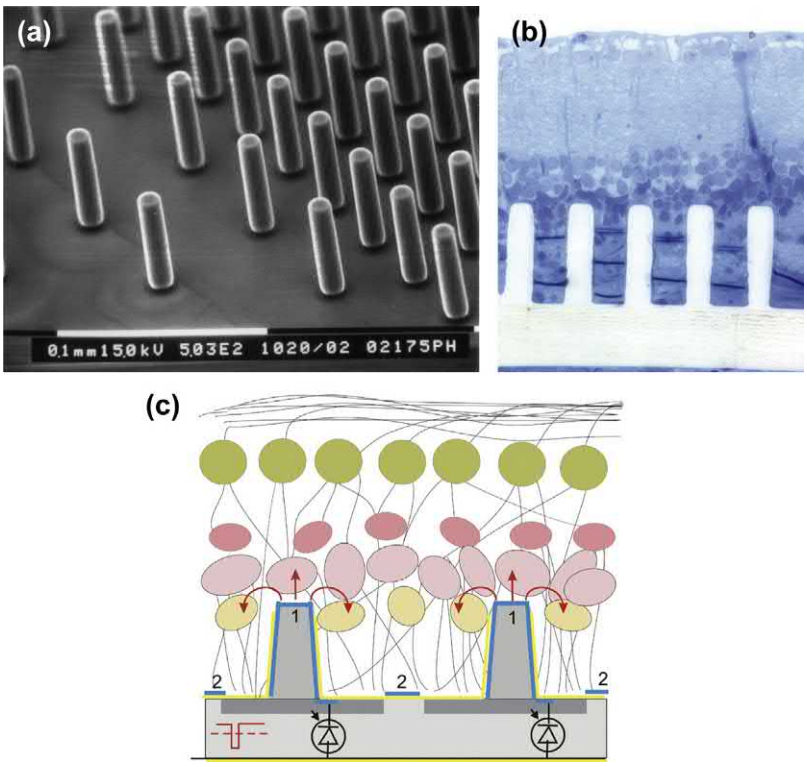

FIGURE 63.6 Pillar electrodes penetrating into the middle of the inner nuclear layer of degenerated retina. (A) Electron micrograph of an array having pillars of 10 μm in diameter and 70 μm in height. (B) Histology of the RCS rat retina with a pillar array 6 weeks after implantation. (C) Schematic of a retinal prosthetic: retinal cells in the inner nuclear layer stimulated by pulsed electric fields emanating from the exposed tops of the pillars. *RCS*, Royal College of Surgeons. *Images provided by Daniel Palanker, Ph. D., Stanford University.*

that code for proteins reacting to certain wavelengths of light by depolarizing or hyperpolarizing the cell membrane. The two primary proteins used in this research are channelrhodopsin-2 (ChR2) and halorhodopsin (NpHR), although melanopsin [95] is also considered a candidate for gene therapy.

Neuron depolarization is achieved by stimulating ChR2. This protein is a microbial-type rhodopsin from the green alga, *Chlamydomonas reinhardtii*. Nagel et al. [96] found ChR2 to be a plasma membrane channel that passively conducts both mono- and divalent cations upon capture of a photon by its attached chromophore. Similar to melanopsin ($\lambda_{max} = 480$ nm), ChR2 has an absorption peak at approximately 460 nm [96].

Conversely, neuron hyperpolarization can be achieved by stimulating NpHR. Originally thought to be an outward-directed cation pump, Schobert et al. [97] identified halorhodopsin as an inward-directed Cl^- pump. Although these experiments were performed on halorhodopsin from *Halobacterium halobium*, a variant (NpHR) from *Natronomonas pharaonis* is used for current experimentation because of its higher affinity for extracellular Cl^- [98,99]. Both forms of halorhodopsin absorb light at longer wavelengths than ChR2, with λ_{max} at approximately 580 nm. The two labs simultaneously reported the use of NpHR to optically hyperpolarize neurons.

These tools can be delivered to retinal cells by a variety of methods. The most widely used delivery system is that of adeno-associated virus type 2 (AAV2) vectors. These vectors were first reported for human preclinical gene therapy trials by Flotte et al. [100], applied to cystic fibrosis. Since then, the use of AAV2 for human gene therapy has expanded to cover a number of conditions [101]. Encouragingly, Simonelli et al. [102] reported the use of AAV2-based treatment for LCA with maintained single-dose efficacy and no serious adverse events as of 1.5 years posttreatment.

Bi et al. [92] injected AAV2, loaded with ChR2/GFP coding attached to a strong ubiquitous promoter, into the intra- and subvitreal spaces of mice. The authors found expression of the ChR2/GFP chimera in ganglion, amacrine, horizontal, and to a lesser extent, bipolar cells of the retina. Light responses were confirmed by whole-cell patch clamping in retina slices and visually evoked potential (VEP) recordings from the visual cortex of otherwise blind mice. These authors further demonstrated that even nonspecific transduction of ChR2 in degenerated retinae can produce light responses in the visual cortex. The quality of such information reaching cortex, however, may not be particularly meaningful. Specifically, the simultaneous activation of pathways that normally exhibit direction selectivity, lateral inhibition, and ON, OFF, or ON–OFF

responses would likely generate a highly corrupted rendition of the original images.

One way to avoid such signal corruption would be to limit the classes of cells that receive photic input. Busskamp et al. [103] reported transducing surviving cone cell bodies with eNpHR [104], a variant of NpHR with improved cell membrane localization. The target cones were in mouse models of RP and in human ex vivo retina explants. In mouse models the authors demonstrated that such cone transduction could create light responses in the retinal network that displays lateral inhibition, direction selectivity, and ON and OFF responses. Live mice with transduced cones also displayed visually guided behavior. In human retinal explants, the authors showed that their AAV2 delivery method successfully transduces human cones and results in the generation of photocurrents indicative of eNpHR activity. Although cone transduction would not be a viable treatment for patients with too few remaining cones, the authors identified an existing population of RP patients that had some remaining cone cell bodies, despite the apparent loss of their outer segments.

Another set of targets for cell-specific transduction in the retina is ON bipolar cells. While there is no promoter that is known to specifically target OFF bipolar cells [103], ON bipolar cells can be targeted using the promoter for the gene encoding the metabotropic glutamate receptor, mGluR6 [105,106]. Although not as advantageous as stimulating the retinal network from the level of the cones, specifying ON bipolar cells should allow for signals far less corrupted than one would expect from indiscriminate stimulation. Doroudchi et al. [105] used AAV2 to transduce ON bipolar cells in mouse models of blindness with ChR2. The authors observed ON-type RGC responses and visually guided behavior in the test animals. Unfortunately, when Fradot et al. [107] performed a similar experiment in human retina explants, only a very small fraction of ON bipolar cells was transduced. More efficient promoters and/or delivery methods will need to be explored before useful, selective transduction of ON bipolar cells can be realized [93,107].

While AAV2 delivery has been shown to be very productive, a number of researchers are doubtful of its practical use in the clinic. Specifically, the use of viral vectors may introduce its own risk of immune responses, and it imposes limits on the size of DNA packages to be delivered [108]. Parallel to research using AAV2 vectors are experiments that create pores in target cells through which desired genetic material can be absorbed. These pores can be generated using electric fields [109] or a femtosecond-pulsed laser beam [110].

In 2008 Lagali et al. [106] used electroporation to transfect ON bipolar cells of mouse models of retinal degeneration. Actually predating the analogous AAV2 experiments, these authors also observed expected ON ganglion cell responses and optomotor behavior, as well as center-surround organization and cortical responses. There is no indication, however, whether electroporation would have any more success than AAV2 at transfecting human ON bipolar cells. More recently, Gu and Mohanty [111] also transfected ganglion cells with ChR2 in mouse models of retinal degeneration, generating visually guided behaviors.

Electroporation, by its nature, acts over a relatively wide area and does not offer more spatial specificity than AAV2 vector delivery. Should the treatment be aimed at specific parts of the retina, as in RP patients with remaining central vision, a more focal method of transfection may be desirable. By applying a 10^{12} W/cm 800 nm laser beam using femtosecond pulses, researchers can create transient perforations in specific cells through which DNA can enter [112].

Gu and Mohanty [111] used this optoporation to transfect RGCs of goldfish with ChR2. Using multiphoton targeting, the authors were able to specify the 3D location of each pore, which had radii of approximately 400 nm and was laid out in 3 μm intervals over the targeted area. In a follow-up study, Villalobos et al. [110] transfected retinae of the same species using both optoporation and photothermal poration with carbon nanoparticles. Stimulation through ChR2 was observed by intracellular calcium imaging.

Separately from these experiments on targets and delivery methods, Nirenberg and Pandarinath [113] recently addressed the issue of optogenetic signal fidelity using a computational approach. Unlike other groups discussed here, these authors bypassed the typical issue of transfection by growing a transgenic mouse line that models retinal degeneration and possesses ChR2-expressing RGCs. To determine proper stimulation parameters for these ganglion cells, the authors observed the spiking patterns of different classes of ganglion cells from normal retinae in response to movies with both natural and artificial scenes. Using these data, they constructed encoders, specific for each type of ganglion cell, which combined to model the signal processing performed by the normal retina. The models were data driven, in that parameters for each model were chosen so the output for any given input would have the highest probability of matching actual retinal spike trains, as tested by cross-validation.

Nirenberg and Pandarinath stimulated the retinae of blind mice using either patterns generated by their encoder or the unprocessed spatial patterns of the stimuli. The authors demonstrated that cells stimulated by the encoder's patterns more closely matched the firing patterns of normal retinal neurons. Further, the authors used a drifting grating on an LCD screen to elicit optomotor tracking, which was only possible when the stimulus was

presented though the stimulation encoder. No tracking was observed with the unprocessed stimulus or in control animals. These data imply that, although cerebral cortex is plastic, meaningful perception of visual information may depend on the preservation of the neural code normally exhibited by the retina.

For clinical purposes, Nirenberg and Pandarinath acknowledge that it may not be practical to target multiple classes of ganglion cells, each receiving its own coded version of a given stimulus. They do argue, however, that targeting a single ganglion cell class, with proper encoding, may be sufficient to provide more faithful percepts than those generated by conventional stimulation patterns. Their experiment inducing optomotor tracking, in fact, only utilized transient ON cell encoders.

Once applied to human subjects, optogenetic approaches to vision restoration may outpace less target-specific electrical retinal processes. Reactivation of cone remnants using NpHR could provide many benefits related to intrinsic retinal processing. For patients in whom such treatment would not be viable, stimulation of bipolar cells, or of ganglion cells in the fashion described by Nirenberg and Pandarinath, could provide similar benefits. To introduce light-sensitive properties into the target cells, electro- and optoporation may offer new avenues of treatment, should cases arise in which AAV2 vectors are insufficiently effective or specific to use as a delivery system.

Outer retinal cell transplantation

Despite almost two decades of careful investigation, reliable, and widespread formation of synapses between transplanted photoreceptor precursors and native inner retinal cells—and thus full integration of the transplanted cells into the host retina—has remained elusive. Also, the effects of the host immune response on graft survival—especially of transplanted RPE—cannot be ignored, even if it appears to be mild, and this host reaction will have to be effectively controlled without long-term systemic immune suppression. Both areas can profit from tissue-engineering approaches. A complex set of conditions—cells in the proper stage of development, properly matched to the host retina, and spurred on by a proper combination of neuroprotective and neurotrophic modifications to the host environment—needs to be met for successful graft−host integration. Recent reports indicate that even widely held beliefs regarding the proper developmental stage of the transplanted cells may need to be revisited. In a mouse model, early postnatal photoreceptors showed widespread synapse formation with native retinal cells [114], whereas most previous research has been directed at the use of postmitotic photoreceptor precursors in a much earlier stage of development.

Even if the cell population affected by the primary degeneration process is successfully replaced, secondary degeneration stages—of RPE cells in photoreceptor degenerative disease and of photoreceptors in RPE degeneration—may still prevent restoration of visual function. This problem can be addressed adequately only by performing a combined graft of RPE and photoreceptors, presumably prepared in a tissue culture environment and stimulated toward integration through a carefully tuned combination of neuroprotective and neurotrophic factors. Culturing stem cells or differentiated cell lines might allow the creation of heterogeneous structures such as RPE/photoreceptor double sheets. Culture conditions and the growth stage of these sheets should be modulated to prepare the cells for integration with the host retina. In order to provide sufficient structural support to these fragile sheets during growth and transplantation, a resorbable polymer layer could be used as a substrate.

Cell matrices supporting axonal regrowth

Cell culture conditions and retinal integration alone will not be sufficient in the case of ganglion cell transplantation with the objective of repopulating the optic nerve with axons carrying visual information to the brain. As discussed earlier, this will require the (re)creation of conditions favorable for axonal growth over distances of many centimeters and the ability of sprouting axons to contact target cells with the correct retinotopic mapping. This feat is accomplished effortlessly by RGC axons in the developing embryo, but to recreate the conditions for this to occur in an adult organism will be a considerable challenge.

As was noted earlier, an RGC axon damaged by glaucoma, optic nerve disease, or injury can only form new neurites under optimal environmental conditions, which essentially mimic those in a developing organism. At the same time, neuroprotective factors are required to sustain the RGC long enough for the axon to assume this support function. If the glaucoma diagnosis is made early, and safe and effective neuroprotective agents become available, it may be possible to save most of the threatened axons and thus spare most RGCs and the patient's vision. In practice, however, many axons may have been lost by the time the diagnosis is made, and protection of the cell somata and stimulation of axon regrowth become the treatment objectives [115]. The experimental conditions employed thus far, using peripheral nerve sheaths to create a substrate for axonal regrowth, are less than ideal, because they do not provide an integrated environment in which regrowing axons combine with intact remaining axons and in which protection of the threatened RGCs is built into the environment. This is a very active field of research, offering great potential for tissue-engineering

approaches, and one may expect such approaches to play a major role in achieving these objectives. Understanding the necessary conditions to create an integrated environment for axonal regrowth and creating novel synthetic materials to provide these conditions should provide wonderful challenges to tissue engineers.

One approach to such an integrated solution might be that engineered cells could be grown in situ, as a loose skeleton of supporting tissue, to follow the course of the optic nerve to the chiasm and optic tract; these cells would be programed to exude the necessary factors promoting axon growth and RGC protection. Alternatively, it might be possible to modify the normal environment of the optic nerve (temporarily) to allow or even stimulate axonal growth. Assuming that it would be possible to guide outgrowing axons through the optic chiasm toward the appropriate structures and retinotopic projections in the midbrain, RGCs may restore their connections to the target cells and resume functional visual processing.

Repopulating ischemic or diabetic retina

As mentioned earlier, new capillaries—formed under the influence of an angiogenic tissue response—tend to be poorly organized and fragile, causing leakage and thus a great deal of damage to already stressed retinal tissue. Therefore the prospects of restoring vision in such retinal areas are, at the present time, poor.

This may change, however, if cell populations can be grown in vitro and introduced into the retina under physiological conditions mimicking those during embryonal development. In that case the formation of new blood vessels could follow a more orderly pattern, and implanted cells would have a much better chance of forming functional connections. Whether and when it will be possible to recreate embryonal conditions and grow such integrated retinal tissue as is required to restore vision to an ischemic portion of the retina, from RPE to RGC axons, is difficult to predict. It is a challenge whose magnitude exceeds that of RPE/photoreceptor transplants, functional stimulation of inner retinal cells, and RGC protection/axonal regrowth combined.

Assessing the functional outcomes of novel retinal therapies

Electrophysiologic assessment of the functionality of a therapeutic intervention is considered a gold standard by many researchers and clinicians, but recording the responses in eyes that have undergone experimental treatments may not always be feasible. Retinal implants are in a unique position that they allow clinical functional evaluation directly from the implant provided that the necessary signal collection hardware is available. This is exemplified by developments in cochlear implants. Cochlear implants were approved for clinical use in the United States in 1984 [116], and over the years advanced built-in electronics based on reverse telemetry [117] have been developed to record electrically evoked auditory nerve responses (electrically evoked compound action potentials, or eCAPs). eCAPs are recorded by stimulating the auditory nerve through a subset of electrodes in the implant array and recording the elicited neural activity at a more distant electrode. The recorded data is sent back through the same wireless radiofrequency link that is used to stimulate the implant. eCAP recordings are nowadays routinely applied intraoperatively [118], enabling the surgical team to assess functionality of the implanted device while the patient is still under general anesthesia. In addition, eCAPs can be used for rehabilitation purposes such as automated device fitting [119,120] and fitting of cochlear implants in young children [121,122].

The visual equivalent of the eCAP is the electrically elicited electroretinogram (eERG), which could fulfill similar purposes to the eCAP. However, none of the present retinal implant systems, such as the Argus II device, has reverse telemetry capabilities sufficient for recording the eERG. Recording the eERG with standard ERG methods such as corneal electrodes is difficult, due to the small signal amplitudes and the presence of electrical as well as physiological artifacts in the eERG signal [123]. Electrical artifacts generated by the implant can be effectively reduced by using filtering techniques such as stationary wavelet transformation [123–126]. Physiological artifacts are more difficult to deal with, because filtering techniques reducing these contaminations will inevitably also reduce the retinal responses of interest. In a recent conference presentation reporting on recordings from monocularly implanted Argus II recipients [123], it was demonstrated that a physiological artifact attributable to pupil innervation responses in both eyes can be largely eliminated through subtraction of the contralaterally recorded pupil response from the combined retinal + pupil response recorded at the implanted eye (Fig. 63.7). Although the decontaminated corneal recordings have negative polarity, as seen in the eERG of rats with retinal implants [127], and show increasing amplitude with increasing stimulus level as expected from any compound action potential, their retinal origin remains to be definitively confirmed. It is likely that this confirmation will have to await direct recording, with high temporal and dynamic resolution, from implant electrodes through improved telemetry.

In the interim, cortical responses, that is, electrically elicited VEPs (eVEPs) recorded from the scalp, can provide a promising alternative [128,129] to retinal responses (Fig. 63.8). Cortical [130] and brainstem [131] responses have been used to assess cochlear implant function, but

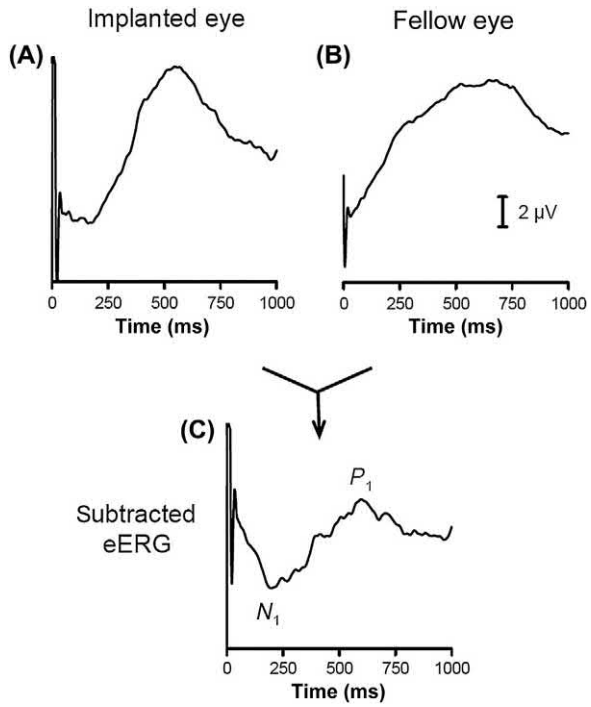

FIGURE 63.7 Electrically elicited electroretinograms (eERGs) evoked by unilateral stimulation of the implanted eye and recording the eERG bilaterally (A and B). The decontaminated eERG (C) was obtained by mathematically subtracting the physiological artifact recorded from the fellow eye. *eERGs*, Elicited electroretinograms.

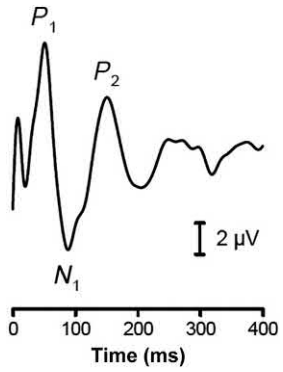

FIGURE 63.8 Cortical response of retinal implant stimulation: the electrically elicited visually evoked potential (eVEP).

eCAPs are applied more widely, due in no small measure to the availability of high-quality reverse telemetry systems [132]. Nevertheless, advantages of cortical over peripheral potentials include the fact that cortical responses represent central nervous system activity (V1 in case of eVEPs) and may provide a more accurate indication of perception than eERGs. In addition, eVEPs can also be recorded in case of visual prostheses implanted in the optic nerve [133] or central nervous system; are relatively easy to acquire; show the expected dependence on stimulus level; and accurately reflect subjective threshold [129]. Unfortunately a single eERG or eVEP recording can take up to 15 minutes to complete, using current technology, thus limiting the usefulness of this approach at the present time. For the eERG and eVEP to be of clinical value, recording times will have to be substantially reduced; once this is accomplished, these recordings can be used for system fitting in a similar fashion as is currently the case for eCAPs in cochlear implants.

Conclusion: toward 2020 vision

The potential applications of tissue engineering sketched in the previous sections pose enormous challenges, well exceeding the competency of any single group or institution. Concerted research efforts by multidisciplinary groups may allow the implementation of the complex systems required to restore and enhance vision. As researchers improve, on the one hand, their fundamental understanding of processes such as photoconversion, graft integration, immune regulation, and neuroprotection and, on the other, the engineering ability to control tissue properties and neurite growth, both in vitro and in situ, crude but functional vision restoration at the RPE/photoreceptor level and at the RGC level may advance to the level of experimental and even clinical therapy. Integration of all these areas to recreate the full range of retinal processing is a much more distant goal, for which the header of this section is much too ambitious.

To accomplish any of these forms of vision restoration, however, funding mechanisms for multidisciplinary research and interest from the corporate sector will have to rise well beyond their current levels. While the number of severely visually impaired individuals and the economic impact of vision restoration alone may not justify that these approaches receive priority over the treatment of life-threatening conditions, the investment required is relatively modest, and the improvement in quality of life for (nearly) blind patients can be very significant.

Acknowledgment

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Further reading

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Part Seventeen

Oral/Dental applications

Chapter 64

Biological tooth replacement and repair

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Introduction

Teeth might not at first seem to be an obvious organ for developing biological methods for replacement or repair. Teeth are nonessential organs, and modern dental treatments enable most dental problems to be treated using traditional nonbiological approaches. Teeth do, however, offer unique opportunities to develop tissue-engineering-based approaches on an organ that is not only easily accessible and nonlife-threatening but one where there are a large patient numbers and a significant clinical problem. The challenges of developing methods needed to restore and repair complex organs are immense, but the biggest challenge will be the testing of any such organs on patients. Patients requiring repair or replacement of major internal organs (heart, liver, lungs, pancreas, etc.) are by definition likely to have a serious illness. Access to these organs requires major surgery; should the treatment fail, the consequences will be severe and life-threatening. Teeth do not present any such problems and thus provide an opportunity for the proof of concept to be tested safely, with little chance of danger to the patients.

Although there are an increasing number of alternative treatments in dentistry, most are nonbiological and many are based on techniques that have been practiced for thousands of years. Dental implants, for example, involve the replacement of missing teeth with metal rods that are screwed into holes drilled into the jawbone. The practice of replacing missing teeth with metal implants can be traced back to the ancient Romans and Egyptians [1,2]. The number of dental implants installed is increasing each year, and thus there is a need to develop new biologically based approaches. Similarly, many tooth fillings still use mercury-based amalgams, and the possibility that repair of dental hard tissues (dentine and enamel) might involve the use of cells to remineralize the damage to teeth naturally is an exciting prospect. This chapter explores the current status of research directed toward whole tooth replacement and repair of dental disease.

Tooth development

Teeth are ectodermal appendages (hair, sweat glands, salivary glands, etc.) that develop from an increasingly well-understood series of reciprocal epithelial mesenchymal interactions (Figs. 64.1 and 64.2). In mammals, these interactions take place in the developing oral cavity between the oral ectoderm (epithelium) and cranial neural crest-derived mesenchyme (ectomesenchyme). The cellular origins of the signals that initiate tooth development have been the subject of much controversy over the years, but modern molecular and animal techniques have confirmed that the oral (predental) epithelium is the source of the signals that initiate odontogenesis (reviewed in Refs. [3,4]).

Recombination experiments between dental cells and nondental cells have identified temporal changes in the direction of inductive signals. Following the initial epithelial-to-mesenchymal inductive signals, a long series of temporally and spatially controlled exchange of signals governs each step in the increasingly complex development of a tooth. With the exception of the nerve supply, all the cells of a mature tooth originate from the oral epithelium and ectomesenchyme. The epithelial cells form only one functional cell type, the ameloblasts that are responsible for enamel formation. All of the other cell types, including the odontoblasts that are responsible for dentine formation, periodontal ligament cells, the pulp cells, etc., are derived from ectomesenchyme. The ability of ectomesenchyme to differentiate into these different cell types illustrates the stem cell–like properties of these cells. Classic recombination experiments in the 1980s established that nonneural crest cell populations of mesenchymal cells cannot respond to tooth-inductive signals,

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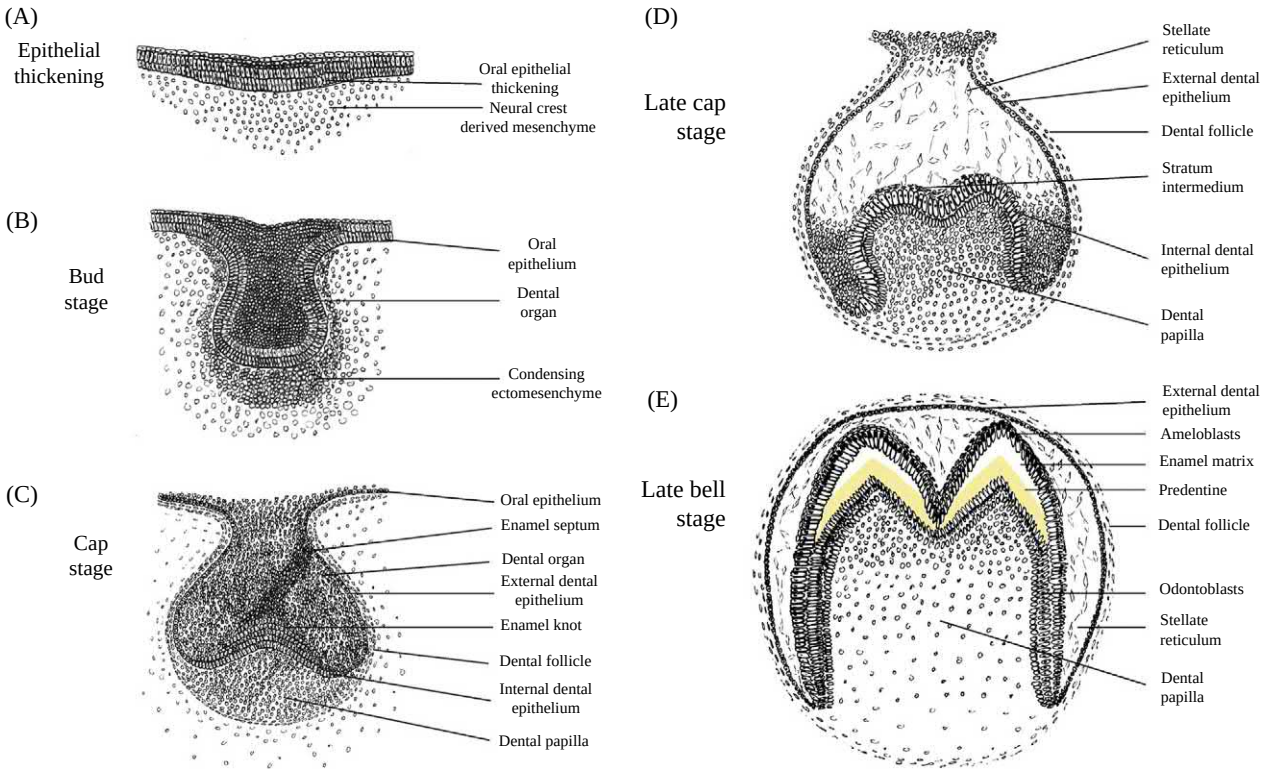

FIGURE 64.1 Drawings of histological sections of mammalian first-molar-tooth development, from the epithelial thickening stage (A) through to the late bell, stage (E).

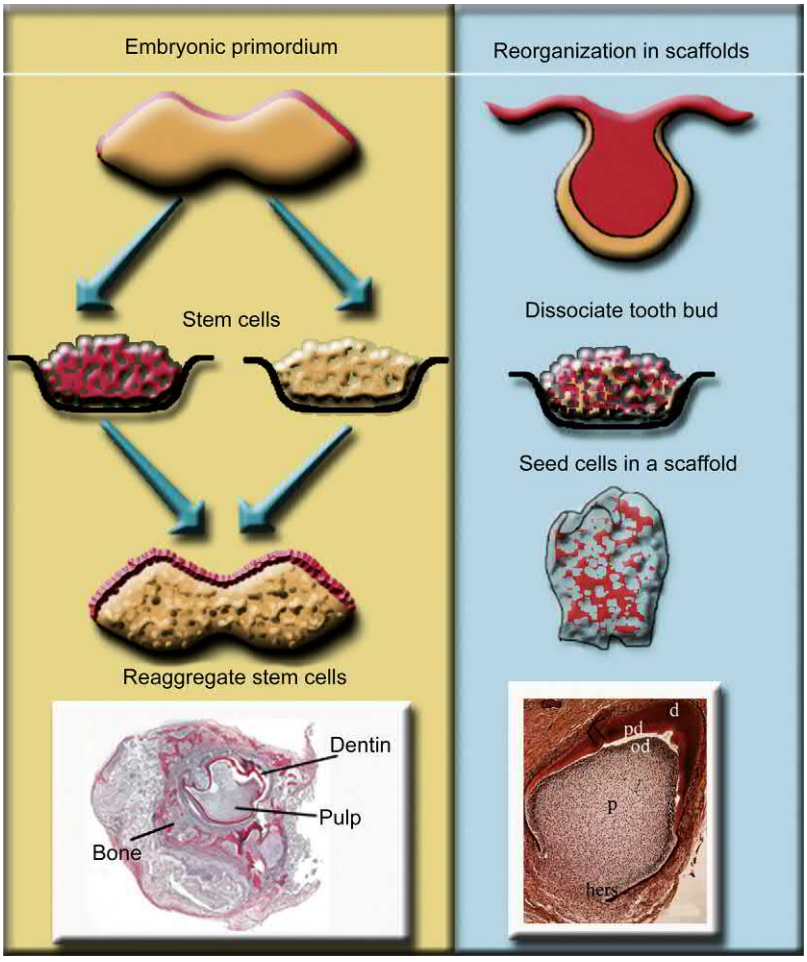

FIGURE 64.2 Diagrammatic representation of two methods currently being explored for producing biological tooth replacement. *Figure kindly drawn by Rachel Sartaj.*

and, more recently, the requirement for dental mesenchymal cells to have stem cell properties has been established (see later). An understanding of the properties of predental epithelium, mesenchymal cells, and the interactions and molecules involved is the key to developing biological approaches for replacement and repair.

Whole tooth-tissue engineering

Stem cell-based tissue engineering of teeth

The aim of this approach is to reproduce an embryonic tooth primordium from cultured epithelium and mesenchymal cells. Since it is established that embryonic tooth primordia are able to continue their embryonic development in ectopic adult sites such as the kidney, the expectation is that the artificial primordia will develop into teeth following implantation as tooth rudiments into the adult mouth. Thus rather than a metal dental implant, a cell-based biological implant will be transplanted. Since the ectomesenchymal cells of the tooth primordium form the majority of tooth cells and regulate tooth shape, it is finding replacements for these cells that has been the main initial focus of research. The early inductive interactions that take place in vivo show that the mesenchymal cells must have stem cell−like properties. Cultured populations of stem cells have thus been used to replace these ectomesenchymal cells in recombinant tooth explants. Cultures of mouse embryonic stem (ES) and neural and adult bone marrow cells were aggregated to form a semi-solid mass, on which embryonic oral epithelium was placed [5]. When cultured for 2−3 days, the initiation of an odontogeneic response in the stem cell "mesenchyme" cells could be visualized with molecular markers. Transfer of the explants in the kidney capsules of adult mice was then used to assay for tooth formation. "Bioteeth" explants made with mesenchyme derived from ES and neural stem cells proved difficult to transfer intact into kidneys. Explants made from adult bone marrow stromal cells, however, were substantially more robust and survived transfer. Following incubation of explants in kidneys for 10−14 days, clearly identifiable tooth crowns were formed, surrounded by bone. By using cells from genetically distinct transgenic mouse lines (GFP, LacZ), the stem cell origins of dental mesenchyme cells and bone cells could be confirmed. Cells derived from adult bone marrow stromal were thus capable of contributing to tooth formation is a way identical to ectomesenchyme cells. Moreover, development of the tooth crowns appeared to follow the normal pathway of embryogenesis, with the formation of new bone completely surrounding the tooth. New bone formation is an essential (but often overlooked) component of biotooth formation. A biotooth has to be able to anchor itself to the jawbone with roots and a periodontal ligament. Concomitant bone formation must therefore occur during tooth development.

The growth of tooth primordia following surgical transplantation into the adult jaw appears remarkably straightforward. Transplantation into ectopic sites or into tooth sockets following extraction provides a suitable environment to support continued development, postnatal growth, root formation, and eruption [5−7].

Bioteeth from cell-seeded scaffolds

In the early 1950s, Glasstone Hughes demonstrated that when early-stage embryonic tooth primordia are physically divided into two halves; each half develops into a normal-sized tooth [8]. This pioneering experiment demonstrated the developmental plasticity and regenerative capacity of embryonic tooth germs. This regenerative capacity has been exploited to devise a simple, biodegradable scaffold-based approach to biotooth generation. The early pioneering work of Vacanti et al. on the use of biodegradable scaffolds to act as supports for guided tissue regeneration has provided the basis for using the reorganizational properties of dental primordia cells to reform teeth in vitro [9]. The basic principle is to create scaffolds in the shape of the tooth required. These are then seeded with cells isolated following dissociation of third-molar-tooth germs. Third molars, or wisdom teeth, erupt late in human development and thus in young adults are present as dormant primordia. Both pig and rat third-molar primordia have been used with essentially the same procedure, utilizing biodegradable polymer scaffolds [10−12]. Cells from third molars at the late bud stage of development were dissociated by incubation with collagenase and dispase, and the cells were then either seeded directly into scaffolds or cultured for up to 6 days in Dulbeccos modified eagle medium plus 10% fetal bovine serum before seeding. The scaffolds were composed of polyglycolate/poly-L-lactate and poly-L-lactate-*co*-glycolide prepared using polyvinyl/siloxane and molded into the shape of human incisors and molars. For pig cells, scaffolds of $1 \times 0.5 \times 0.5$ cm were used, whereas for rat cells, rectangular scaffolds of $1 \times 5 \times 5$ mm were used. Scaffolds containing seeded cells were surgically implanted into the omentum of rats (athymic rats for pig cells) and left for 12−30 weeks. Histological sectioning of the explants revealed the formation of tiny tooth-like structures. The structures were between 1 and 2 mm in size and showed many of the features of molar-tooth crowns, including differentiation of ameloblasts and odontoblasts. Experiments using pig cells detected the tooth-like structures after 20 weeks, whereas with rat cells, these formed after 12 weeks. In all cases the shapes of the toothlets formed were independent of the shape of the scaffold, and, unlike natural tooth formation, no bone was formed in

association with the teeth. Based on the previous results of Glasstone Hughes, the most likely explanation for these results is one of reorganization rather than de novo formation. The small size of the teeth indicates that small numbers of dental epithelium and mesenchyme cells reassociated to form tooth germs. The fact that cells could be cultured for up to 6 days indicates that either the epithelial and/or mesenchymal cells are able to retain their odontogenic properties, or the possibility exists that stem cells were present in the cell populations, but this remains to be demonstrated. A functional biotooth must be able to form roots; in order to do this, new bone must form at the same time as the tooth. In the scaffold approach, no new bone is formed. In order to address this, scaffold tooth-like structures have been generated together with bone implants produced from osteoblasts induced from bone marrow progenitor cells seeded onto polyglycolide-*co*-lactide-fused wafer scaffolds [13]. The codevelopment of bone and tooth-like structures permitted the early formation of roots and thus demonstrated the possibility of utilizing a form of hybrid tooth/bone-tissue-engineering approach.

The phenomenon of reorganization of dental primordia cells into teeth has been investigated in detail by Lesot et al. Using cap-stage-tooth germs (E14—mouse) (Fig. 64.1), they showed that following complete dissociation of both the epithelium and mesenchymal cell derivatives, teeth could be produced when the cells were reaggregated and reassociated [14,15]. Thus as observed with third-molar-tooth primordial, the cells retained their odontogenic capacity following dissociation. This property was utilized as a method of investigating the potential of nondental cells to participate in tooth development. The experiments of Ohazama et al. [16] showed that bone marrow stromal cells were able to replace all dental mesenchyme cells. Bone marrow cells were mixed with dissociated dental epithelial cells and reassociated with dental mesenchyme [17]. The bone marrow cells were found to contribute to ameloblast formation. In order to determine which cells within the crude marrow population might give rise to epithelial ameloblasts, cell sorting with c-kit was used to separate hematopoietic progenitor cells. When mixed with dental epithelial cells, these cells were observed to form both ameloblasts and odontoblasts. The ability of bone marrow mesenchymal progenitors (stem cells) to contribute to ameloblast formation was not assessed in these experiments. However, the principle, as originally proposed by Ohazama et al. [16], that bone marrow cells can be a potential source of cells for tissue-engineering bioteeth is further supported by these reaggregation experiments. In all the experiments reported by Lesot et al. the ability of nondental cells to contribute to tooth formation occurs only when the cells are mixed with dental cells from cap-stage tooth germs. The experiments reported by Ohazama et al. [5] did not require a mixed population. It remains to be seen therefore whether any commercial biotooth procedure could feature the use of dissociated embryonic third-molar dental cells. Since bone marrow can be used totally to replace dental mesenchyme in the absence of any dental mesenchyme cells, the prospect exists that if a source of epithelial cells can be identified that can replace dental epithelial cells in the absence of any epithelial cells, then bioteeth could be formed entirely from adult cells. Dental epithelial cells produce the signals that initiate the whole process of tooth formation, and, thus, a nondental source of these cells must in part be capable of reproducing these signals.

One signal that has been identified is bone morphogenetic protein (BMP)4, which is specifically expressed in early predental epithelium before bud formation. When exogenous BMP4 is added to nondental embryonic explants, odontoblast and ameloblast differentiation can be detected [16]. Although these cells are organized together in cell layers as they appear in vivo, they are not present in a recognizable tooth structure. Nevertheless, the fact that one molecule applied to cells early can stimulate the differentiation of nondental cells into both epithelium and mesenchymal dental cells offers the possibility of engineering a dental epithelium from nondental epithelial cells.

Root formation

In order to be functional, bioteeth must develop roots. Root formation is a complex and little-understood process. However, although little is known of the molecules that stimulate and coordinate root formation, the absolute requirement for bone is established. If teeth form in the absence of bone, they cannot form roots, as observed in the seeded scaffold tooth-like structures. When these scaffold teeth develop alongside new bone, root formation is stimulated [13]. Tooth formation is most often studied experimentally by the transplantation of tooth primordia to ectopic sites in adult rodents. The anterior chamber of the eye, the kidney capsule, the omentum, and the ear have all been used as sites that permit tooth formation. Root formation can occur at all of these sites if accompanied by bone development [17]. A prerequisite of any method of biotooth formation therefore is that tooth primordia are able to form teeth and roots integrated into jaw bone in the mouth. The mouth is, however, not routinely used as an experimental ectopic site for tooth development because of the difficulty of the surgery involved. In order to determine whether the adult mouth can support tooth formation, embryonic tooth primordia have been surgically transplanted into the mouths of adult

mice. Not only do these develop into teeth in the mouth, they also produce functional roots and erupt [5].

Cell sources

The biggest hurdle to a successful clinically translatable protocol for whole tooth-tissue engineering is the issue of the source(s) of cells to be used. Both epithelial and mesenchymal cell sources need to be identified and with the exception of the use of bone marrow stromal cells, all other reports of whole tooth "bioengineering" have employed cells dissociated from tooth primordia. A feature of the successful experiments using dissociated tooth germ cells is that the cells are not expanded in vitro before use. In order to obtain sufficient cells to generate one tooth, many tooth primoria need to be dissociated to produce at least 5×10^4 cells [18]. For any viable clinical approach the cells, whatever their source, will have to be expanded in vitro. However, when tooth primordia cells are expanded in vitro, they rapidly lose their ability to reparticipate in tooth formation, and adult dental cells do not retain any tooth-inductive capacity [19,20]. A major challenge therefore is to develop ways of maintaining tooth forming capacity following in vitro cell expansion.

Since tooth formation requires both epithelial and mesenchymal cells and these cells exhibit reciprocal signaling interactions, one of the cell types used must be able to induce odontogenic capacity in the other. In the bone marrow stromal cell experiments, embryonic tooth epithelium was used to induce this capacity in the bone marrow–derived cells [5]. Cells isolated from adult oral mucosa are capable of responding to odontogenic-inducing signals from embryonic tooth mesenchymal cells and forming tooth-like structures [21,22]. Potential adult cell sources are thus available, but none have inherent odontogenic-inducing properties.

The key to advancing this approach further therefore is to understand the molecular basis of this loss of inductive capacity. When cultured embryonic mesenchyme cells (noninductive) are mixed with noncultured embryonic mesenchyme cells (inductive) and combined with a responding epithelium, the noninductive cells acquire inductive potential and participate in biotooth formation [23]. This "cell community" effect is cell concentration dependent and provides an opportunity to investigate the gene expression changes that are associated exclusively with inductive capacity.

When tooth primordia mesenchyme cells are expanded in culture, they immediately lose contact with the opposing epithelial tissue (a source of signals) and tight cell contacts are lost as the condensed mesenchyme becomes a single layer of cells. Mesenchymal cell condensation is an important phenomenon that is involved in mesenchyme to epithelium signaling and is required for tooth development beyond the bud stage [24]. Maintenance of cell contacts may be established by growing cells in hanging drops. A method has proved successful for hair dermal papilla cell however the large numbers of cell needed for tooth induction make this method inappropriate [25]. Gene expression screens have been used to identify signaling factors whose expression is lost in cell culture [26]. An issue with these however is identifying loss of inductive signals from all the other gene expression changes associated with monolayer culture and loss of cell contact, etc. Such screens have been successful with hair cells because hanging drops have been used to restore lost inductive capacity. This is a powerful tool since gene expression can be compared between inductive cells, cells that have lost inductive signals, and cells where inductive signals have been restored in hanging drops.

Dental-tissue regeneration

An important long-term goal for dental-tissue engineering is developing strategies for biotooth formation, thereby addressing many of the clinical problems arising from developmental anomalies and dental disease. However, in the shorter term, there are many opportunities to be exploited for partial reconstruction of the tooth organ by tissue regeneration. Such approaches may be more readily achieved and still provide the potential for very significant impact on delivery of oral health care. Natural tissue regeneration, that is, wound healing in the dental environment, is well recognized but represents a rather serendipitous event, and the approaches used to encourage such processes are somewhat empirical. A key factor in encouraging natural tissue regeneration is facilitating a conducive tissue environment in which the regenerative processes can take place, including moderation of chronic inflammatory events, control of bacterial infection, and minimizing tissue injury during any restorative surgical intervention. A good understanding of how to control these influences on tissue regeneration is fundamental to clinical success. There are also significant opportunities for the application of agents, whether directly through bioactive molecules or indirectly through agents that can release tissue-sequestered pools of these molecules locally, to promote regenerative processes and tip the balance between tissue degeneration and regeneration.

Natural tissue regeneration

Natural tissue regeneration implies a cellular basis to regeneration, and the nonvital nature of mature dental enamel provides major hurdles to its regeneration by any but physicochemical remineralization processes. However, the vitality of the dentin–pulp complex provides significant opportunities for regeneration. Regeneration in the dentin–pulp complex will only take place, however, if there is

a conducive tissue environment. Early studies highlighted the causal link between bacteria and inflammatory events in the dental pulp, and the presence of pulpal inflammation provides an effective barrier to initiation of regeneration therein [27]. Thus if natural tissue regeneration is to be facilitated, it is essential that bacterial infection and the consequent inflammation in the tooth are controlled. The inhibitory action of inflammation on regeneration may represent an effect more on signaling events than stem/progenitor cell survival, since inflamed pulp tissue can allow isolation of cells with some stem cell properties [28,29]. The close interplay that is emerging between inflammation and tissue regeneration [30], however, emphasizes the complexity of events in the postinjury tissue environment where intervention should be carefully targeted. Traditionally, bacterial control has often been achieved through extensive surgical removal of infected dental hard tissues, although the trend toward minimal intervention therapy during tooth restoration potentially places the tooth at risk through incomplete bacterial control. Sealing or "entombing" the bacteria within the restoration may help to compromise their viability, as may some of the chemical agents used in the placement of dental materials. The complex relationship between bacteria and inflammation is still emerging as highlighted by the identification of the novel bacterial-killing mechanism termed neutrophil extracellular traps, which localize and kill invading bacteria using antimicrobial peptides and histones, thereby constraining infection progress [31]. Control of pulpal inflammation is critical in the context of tissue regeneration, and an improved understanding of the inflammatory mediators involved [32,33] may allow specific targeting with novel antiinflammatory molecules. Epigenetic approaches offer exciting directions to influence the pulpal inflammation—regeneration balance [34].

The concept of tissue regeneration in the dentin–pulp complex has been recognized since the first report of tertiary dentinogenesis in response to injury from caries by Hunter in the 18th century, and dentistry has long been a pioneer in regenerative medicine through the use of calcium hydroxide to stimulate reparative dentinogenesis to bridge pulpal exposures in the dentin [35]. Tertiary dentinogenesis (reactionary and reparative dentinogenesis are subvariants) represents the upregulation of the dentin-secreting cells, the odontoblasts, in teeth after completion of tooth formation to initiate tissue regeneration in response to injury. With mild injury the odontoblasts underlying the injury survive and respond by upregulation of their secretory activity to form reactionary dentin, while with injury of greater intensity, a number of these odontoblasts undergo necrosis and may be replaced by a new generation of odontoblast-like cells secreting a reparative dentin matrix [36] (Fig. 64.3). Reactionary and reparative dentinogenesis are a consequence of the tissue damage and reflect the intensity of the injury rather than its nature [37].

Importance of the injury-regeneration balance

Dental caries is one of the most widespread infectious diseases globally and continues to be a major health care problem. Initially, colonization of bacteria on the tooth

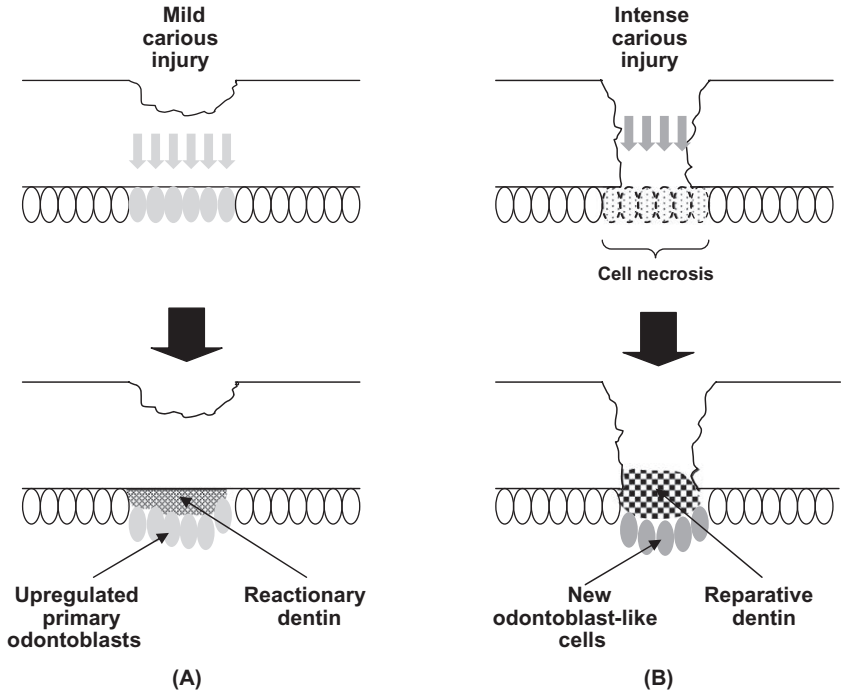

FIGURE 64.3 Schematic diagrams of reactionary (A) and reparative (B) dentinogenesis. (A) Mild carious or other injury signals upregulation of the primary odontoblasts underlying the injury site, leading to secretion of reactionary dentin. (B) More intense injury causes necrosis of the odontoblasts underlying the injury site, and progenitor cells are recruited from the pulp to differentiate into a new generation of odontoblast-like cells, which secrete reparative dentin. Dentin bridge formation at sites of pulpal exposure arises from reparative dentinogenesis.

surface within the dental plaque biofilm leads to demineralization and proteolytic degradation of the dental hard tissues following diffusion of bacterial acids and metabolites. As the disease progresses, both the hard and soft tissues of the tooth become infected, and a sustained bacterial challenge ensues, with consequent host inflammatory responses. The dynamics of the disease process determine the opportunities for tissue regeneration through tertiary dentinogenesis—with a rapidly progressing lesion, little tertiary dentinogenesis or regeneration is seen, but in a more slowly progressing lesion, induction of tertiary dentin secretion occurs immediately beneath the lesion [38]. Classically, this has been assumed to reflect the intensity of microbial challenge, but more recent data indicate that the response is in part due to local release of dentin matrix components. During intense carious injury, appreciable levels of dentin matrix components are released, and these compromise the survival of the odontoblasts [39]. It is possible that growth factors contained within the dentin matrix, particularly those from the TGF-β family, contribute to this loss of odontoblast survival, since levels of TGF-β similar to those released from the dentin matrix have a comparable effect on odontoblast survival in vitro [40]. At lower doses, however, these dentin matrix components and the cocktail of growth factors contained therein can stimulate regenerative events.

It has long been recognized that particles of dentin displaced into the pulp during surgery can act as a nidus for regeneration. Implantation of solubilized dentin matrix components in the base of either unexposed [36] or exposed cavities [41] induces a regenerative response of tertiary dentinogenesis. Such responses mirror those seen beneath more slowly progressing carious lesions [38] and suggest that the natural regeneration seen during carious and other dental tissue injury is induced by local release of bioactive tissue matrix components. If such processes are to be successfully mimicked for development of novel regenerative therapies, it is important that we understand the nature of the signaling molecules involved.

Signaling events in dental regeneration

It seems probable that the signaling events during tertiary dentinogenesis and regeneration in the dentin−pulp complex recapitulate many of those occurring during embryonic development. During tooth development the cells of the inner enamel epithelium of the enamel organ induce the ectomesenchymal cells at the periphery of the dental papilla to differentiate into odontoblasts through the mediation of the dental basement membrane, which may function in the immobilization and presentation of the signaling molecules [42]. Growth factors, especially those of the TGF-β family, may be the key signaling molecules during induction of odontoblast differentiation [43,44]. While an epithelial source of these molecules is not available in the mature tooth for signaling regeneration, sequestration of these molecules in the dentin matrix [45,46] following secretion by the odontoblasts may provide such a source. Their release during carious demineralization [39] by lactic and other bacterial acid metabolites would allow their diffusion through the dentinal tubules to the odontoblasts and pulp cells, which express receptors for these growth factors. Application of recombinant members of the TGF-β family, either in vitro [47−49] or in vivo [50−52], has been demonstrated to induce a regenerative response of tertiary dentinogenesis.

BMP and Wnt signaling pathways play pivotal roles in regulating tooth development [53] and also come into play after pulpal injury. Activation of the Wnt/B-catenin signaling pathway occurs following pulp injury with rapid upregulation of Axin2 with Axin-2 expressing cells going on to differentiate into odontoblast-like cells secreting reparative dentine [54]. Wnt pathway activators, such as glycogen synthase kinase inhibitors, have been demonstrated to stimulate reparative dentinogenesis by activating resident dental pulp stem cells (DPSC) to differentiate into odontoblasts [55] and offer interesting opportunities for the development of novel therapeutic approaches to natural tooth repair.

Although aspects of the signaling events after injury mirror those of embryonic development, their pathological nature means that there is likely to be altered control over this signaling. The activation of signaling pathways seen during repair and the rates of release of molecular signals from the dentin matrix will likely be variable, and the spectrum of cells with which they can interact will differ from embryogenesis.

Control of specificity of dental-tissue regeneration

During wound healing in the dentin−pulp complex a broad spectrum of tissue responses may be observed, ranging from regeneration of dentin tissue virtually indistinguishable from primary physiological dentin in terms of its tubularity and structure to secretion of atubular tissue with many of the structural features of bone. While terms such as tertiary dentin, reparative dentin, and irritant dentin have been used to encompass all of these responses, the dentinogenic specificity of some of the responses remains to be demonstrated. It is unclear, though, as to what the determinants are of the specificity of the response during natural tissue regeneration. Is it control of the molecular signaling processes, is it heterogeneity in the types of cells participating in the regeneration, or is it some other factor? There is still a lack of

consensus on the precise derivation and phenotype of odontoblast-like cells, and it seems probable that the phenotype is broader than sometimes acknowledged [37]. Answering these questions about determinants of response specificity will be fundamental to future strategies for exploiting tissue regeneration in the tooth in a controlled manner. The ability to determine whether tissue regeneration gives rise to tubular or atubular dentin matrix could be of great value in developing designer regenerative clinical approaches. For example, there may be significant benefit in directing secretion of a tubular dentin matrix in the tooth crown during regeneration to restore the normal physiological tissue architecture and function. However, it could be advantageous in possible exploitation of tissue regeneration for endodontic applications, such as root canal therapy, to generate an atubular dentin matrix, thereby providing an effective seal to the periapical region of the tooth.

Dental postnatal stem cells

The cells of the dental pulp have traditionally been considered to be neural crest–derived ectomesenchymal cells, although it is clear that during embryogenesis, the migrating neural crest cells will intermingle with mesenchyme in the first branchial arch [56]. Thus cells of the pulp may not all share the same lineage, although those at the periphery, including the odontoblasts, appear to be of neural crest origin. In reparative situations, there are potentially a variety of possible progenitors in the pulp for differentiation of a new generation of odontoblast-like cells, including undifferentiated mesenchymal cells in the cell-rich layer of Höhl adjacent to the odontoblasts, perivascular cells, undifferentiated mesenchymal cells, and fibroblasts. This highlights the diversity in reparative response, which may occur after injury to the tooth. While the tissues that regenerate in such situations are all referred to as tertiary dentin (reactionary or reparative variants), in reality they represent a spectrum of tissue responses, dependent on the origin of the formative cells [37].

During normal tooth development, it has been suggested that cells destined to differentiate into odontoblasts have to achieve a level of competence before they can respond to an inductive signal for terminal differentiation [42]. If this is the case, it may be a very restricted population of cells able specifically to give rise to odontoblasts during regeneration in the mature tooth. Just prior to terminal differentiation during tooth development, the preodontoblasts align perpendicular to the dental basement membrane, and after the final cell division, one daughter receives the inductive signal to differentiate into an odontoblast, while the other daughter shares a similar developmental history, with the exception of this final inductive step. It has been presumed that this latter group of cells resides in the cell-rich layer of Höhl just beneath the odontoblast layer in the mature tooth and as such, these cells would be prime candidates as progenitors for odontoblast-like cells during regeneration. Certainly, there is a decline in the numbers of cells in this subodontoblastic site with age [57], which mirrors anecdotal clinical reports of impaired tissue regeneration with age. Interestingly, many of the cells in the Höhl layer express the stem cell marker Thy-1 and those cells showing high expression of Thy-1 demonstrated enhanced potential to differentiate into hard tissue–forming cells [58]. Undifferentiated mesenchymal cells are also found within the central core of the pulp, which could contribute to regenerative processes. A specific population of postnatal DPSCs has also been described [59], which show many characteristics of postnatal stem cells, although it is possible that this population of cells may be heterogeneous in its phenotype. Transplantation of these cells subcutaneously into immunocompromised mice in association with hydroxyapatite/tricalcium phosphate powder generated ectopic deposits of reparative dentin with expression of dentin sialoprotein [60]. These DPSCs appear to be distinct from the stem cells from human exfoliated deciduous teeth (SHED) [61] and stem cells from the apical part of the papilla, the latter of which have been isolated from apical areas of the tooth [62]. Despite this focus of looking for specific cell populations in pulp, it is unclear whether these various populations simply represent mesenchymal stem cells (MSCs) recruited through the circulation from sites outside the tooth and if their exposure to the niche environment within the pulp provides their phenotypic characteristics. Genetic lineage tracing provides evidence to support such an origin for some cells involved in odontoblast-like cell differentiation [63], which has significant implications for regenerative strategies in dentin–pulp. Issues with phenotypic change of pulp cells during expansion culture [64] also highlight the opportunities to source stem/progenitor cells from nonpulp tissue, such as bone marrow and adipose tissue [65,66] for dentin–pulp regeneration.

As interest in dental regeneration increases, it is important that detailed characterization be performed of the progenitor cells involved. Primary cultures of dental pulp cells give rise to many cells with myofibroblastic characteristics, and similar results have been observed during extended serial expansion of such pulp cultures [39]. Whether this represents asymmetric growth of these cells or the myofibroblast phenotype represents a default phenotype is unclear, but it is probable that such cells are not true postnatal stem cells, and any attempt to label them as such should be resisted in the absence of fuller characterization. Caution is required in the interpretation of in vitro data though. Pericytes are commonly found in

perivascular locations and can give rise to multiple mesenchymal cell types, including myofibroblasts, when expanded in vitro. In vivo, however, stromal cell differentiation is restricted to tissue-specific cell types, and pericyte populations appear to be molecularly obstructed from differentiating down certain lineages in vivo [67].

Clearly, there is potential for a spectrum of derivations for the cells potentially involved in regeneration of the dentin–pulp complex, and the consequences of this may be a variety of phenotypic responses. It seems probable that only some of these responses may involve true postnatal stem cells. However, the diversity of cellular responses highlights the potential benefits of achieving control over these regenerative processes and the opportunities for developing strategies for directed tissue regeneration.

Directed tissue regeneration

The concept of directing tissue regeneration will be successful only if it builds on the foundation of our knowledge of natural tissue regeneration, which in turn exploits our appreciation of the molecular cellular signaling events during normal tooth development. Focus on both cell- and signaling-based approaches has provided many interesting initial avenues for directed tissue regeneration in the tooth, but it is probable that the combination of these two approaches will prove the most effective in providing us with novel solutions for regenerating tooth structures. For example, seeding of SHED cells in a poly-lactic acid scaffold within a tooth slice has allowed the regeneration of pulp-tissue resembling that seen physiologically [68] and the acidic conditions created by the scaffold were hypothesized to be responsible for release of signaling molecules from the dentin matrix.

Signaling-based strategies

Signaling-based strategies have generally aimed to mimic those signaling events responsible for cell differentiation and secretion during embryonic tooth development and natural tissue regeneration. Thus there has been a strong focus on the application of growth factors, as well as various matrix-derived molecules, which also appear to stimulate regenerative events. Growth factor application has also been investigated for chemotaxis-induced cell homing in pulp regeneration [69], which lends support to the concept of MSC recruitment from nondental locations [63]. Approaches have been used to upregulate secretion by existing cells (reactionary dentinogenesis) as well as to induce the differentiation of new odontoblast-like cells (reparative dentinogenesis) for tissue regeneration, although the former has been constrained by our lack of understanding about the physiological control of odontoblast secretion during primary dentinogenesis.

Both in vitro [47–49] and in vivo [50–52] application of growth factors, particularly of the TGF-β family, to the exposed pulp in pulp-capping situations induces regeneration, although the tissue formed has shown a range of appearances, from osteodentin to tubular dentin-like. There have also been several reports of the application of matrix molecules [70–73] promoting dentin regeneration, and a Phase II clinical trial with a synthetic peptide derived from matrix extracellular phosphoglycoprotein is presently in progress. While all of these approaches provide the foundation for a new era of biologically based regenerative therapies for the teeth, there are a number of limitations. The half-life of free growth factors is generally short; for instance, that of free TGF-β1 is estimated to be of the order of 2–3 minutes. In fact, when the growth factors are sequestered within the dentin matrix, they are remarkably well protected from degradation by association with extracellular matrix components. This protective mechanism can allow the growth factors to remain "fossilized" for the life of the tooth, thereby providing an exquisite life-long potential for natural regeneration. Also, the consequences of short half-life of these molecules and the ubiquitous presence of proteolytic enzymes in the tissue milieu at sites of injury require high concentrations to be applied for regenerative signaling.

Partial proteolytic degradation of signaling molecules sequestered within dentin matrix, however, may in some instances lead to enhancement of their bioactive properties [74]. Implantation of decellularized dental pulp as a scaffold has been proposed for regenerative endodontics [75,76], although we should not ignore that such matrices may also contribute to signaling regenerative events by the presence of bioactive molecules therein.

As for all tissue regenerative and engineering strategies, the mode of delivery of signaling molecules is the key to their effective action. To date, most signaling molecules have been simply applied as lyophilized powders or aqueous solutions, but considerable potential exists for development of novel delivery systems. An alginate hydrogel has shown encouraging results for the delivery of TGF-βs for the induction of de novo dentinogenesis on cut surfaces of pulp tissue [77], and innovative approaches are needed to deliver such molecules through the dentin matrix via the dentinal tubules. While passive diffusion through the tubules will have some effect, histomorphometry of restored human teeth suggests that natural regeneration occurs optimally when there is only a very limited residual dentin thickness, and a challenge will be to develop systems for delivery across greater distances in the dentin matrix. Gene therapy (see later) offers interesting opportunities for targeting signaling molecules

to the regenerative site, although safe and suitable delivery systems are still required.

Calcium hydroxide has long been used to stimulate dentin bridge formation for dental regeneration, although its mechanism of action has remained largely elusive. Recent studies, however, have indicated that both calcium hydroxide, and the closely related material mineral trioxide aggregate, probably act by the release of stores of endogenous bioactive molecules and growth factors sequestered within the dentin matrix, which are responsible for the cellular signaling [78,79]. The limited control of growth factor dissolution from the dentin matrix by calcium hydroxide may in part help to account for its rather variable activity during regeneration. However, its action does point to an interesting approach for regeneration, whereby agents target release of endogenous signaling molecules from the tissue, thereby obviating some of the issues associated with the application of exogenous sources of these molecules. Such agents might also include some of the cavity etchants and irrigants commonly used in restorative dentistry [80,81].

Cell- and gene-based strategies

Cell- and gene-based strategies offer exciting opportunities for dentin–pulp regeneration, although many hurdles have to be overcome before these can become a clinical reality. Using electroporation, Gdf11 gene transfer to an exposed pulp in vivo failed to provide effective regeneration [82], although the use of ultrasound for transfer of the Gdf11 plasmid provided an osteodentinogenic regenerative response [83]. In vivo gene transfer of a recombinant adenovirus containing a full-length cDNA encoding mouse BMP7 failed to induce reparative dentinogenesis in inflamed ferret dental pulps, while ex vivo transduced dermal fibroblasts induced regeneration in the same model, although the dentinogenic specificity of this response requires further characterization [84]. These studies highlight the negative impact on the regeneration of a nonconducive tissue environment, due to inflammation and, also, the limited numbers of stem/progenitor cells in the pulp for recruitment to participate in regeneration. The latter issue might be overcome by the transplantation of suitable stem/progenitor cells for regeneration at sites of injury, especially if transduced with suitable signaling molecules. Ex vivo gene therapy by transplantation of Gdf11 [85] or BMP2—electrotransfected pulp stem/progenitor cells [86]—is seen to be promising for the induction of regeneration, although the tissue formed was osteodentin-like in appearance. It is unclear whether the lack of dentinogenic specificity in these responses can be ascribed to the nature of the cells transplanted or to the inductive signaling molecules. However, the results highlight the importance of considering not only the cells and signaling molecules involved but also the delivery mechanisms for effective therapies to be developed. Novel approaches may also include epigenetic modification of pulp cells with histone deacetylase inhibitors and DNA-methyl transferase inhibitors to stimulate regenerative events [34,87,88].

The anticipated low numbers of stem/progenitor cells for regeneration in the dental pulp indicate the need to optimize recruitment strategies for these cells, whether locally recruited from within the tissue or transplanted from without. A pilot clinical study has demonstrated pulp regeneration after transplantation of pulp stem cells [89] reinforcing evidence from animal transplantation studies [90]. Despite these very encouraging results, challenges still remain both with possible issues of immune reaction to nonautologous cellular material and the introduction of cellular transplantation surgical techniques into everyday dentistry. Targeted stem cell therapy or local cell recruitment require both selection of those cells of appropriate lineage and phenotype and appropriate factors to attract the cells to the injury site. Cell markers for potential selection of stem cells from embryonic first branchial arch tissue have been investigated [91], and low-affinity nerve growth factor receptor is being targeted for the selection of odontoblast-like cell progenitors from the mature dental pulp [39]. A combination of c-kit, CD34, and STR-1 has been reported for selection of stromal stem cells from human deciduous dental pulps, which can be induced to differentiate into osteoblasts for bone regeneration [92], while c-kit has been used for selection of odontoblast progenitor cells from bone marrow [17]. CD105 has been reported to allow for selection of pulp cells capable of regenerating pulp tissue including nerves and vasculature followed by new dentin formation [93]. Use of such cell selection approaches may overcome problems associated with recruitment of stem/progenitor cells for odontoblast differentiation from a relatively small niche within the pulp during dental regeneration. Future strategies might include ex vivo selection of these cells for transplantation or immobilization of antibodies or other capture molecules for selection at regenerative sites for recruitment of the cells. Various bioactive molecules have been demonstrated to recruit stem cells for homing to injury sites [94–96], and their therapeutic use many be envisaged either by direct local application to the injury site or by indirect local release from the endogenous matrix at the injury site using chemical irrigants and tissue-conditioning agents. A combination of these various approaches with novel delivery of signaling molecules may prove effective in optimizing the regenerative response. Use of DPSC-derived conditioned medium also warrants further exploration as a clinical tool [97].

Conclusion

Tissue-regenerative strategies offer exciting possibilities for the development of novel clinical solutions for treatment of dental developmental anomalies and disease. The exquisite natural tissue-regenerative capacity of the dentin–pulp complex in the tooth provides an invaluable foundation on which to develop novel biologically based regenerative therapies. Particular attention will need to be focused on both the control and the specificity of the regenerative processes, and a combination of natural and directed tissue regeneration offers many exciting opportunities for the future.

Progress in the identification, isolation, and understanding of the differentiation of embryonic and adult stem cells, together with a continuing understanding of the control of tooth development, will undoubtedly aid the production and refinement of approaches for biotooth formation. Although there remain many potential problems and pitfalls, biological tooth replacement is now a realistic possibility.

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Chapter 65

Tissue engineering in oral and maxillofacial surgery

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Introduction

The oral and maxillofacial region is defined as the mouth and surrounding structures, bounded superiorly by the cranial base and inferiorly by the lower jaw. It is a highly complex area that includes morphologically intricate skeletal elements (Fig. 65.1), organs responsible for the special senses, lining and covering tissue (i.e., skin and subcutaneous fat), a rich neural and vascular network, and teeth. The prominent position adopted by this area makes it particularly vulnerable to injury, while the complicated interplay of events during embryogenesis of the craniofacial skeleton increases the possibility of developmental aberrations. Regular exposure to various disease-inducing agents, including complex carbohydrates (e.g., sugar), ultraviolet rays, thermal insults, and carcinogens (e.g., tobacco products), produces a variety of common pathological conditions such as dental caries, burns, and malignant neoplasms. The loss of tissue integrity and continuity resulting from trauma, developmental deformities, and pathology imposes upon both physicians and dentists considerable reconstructive challenges. Meeting these requirements in the 21st century will include strategies based upon tissue-engineering (TE) principles. While the discipline itself is several decades old, clinical applications for the reconstruction of the oral and maxillofacial region are currently absent. The purpose of this chapter is therefore to describe the special challenges posed by oral and maxillofacial reconstruction, outline existing reconstructive techniques, and review the available literature on TE protocols that may be relevant. Providing tissue engineers with a description of current reconstructive modalities and, in particular, their shortcomings create a reasonable starting point to base the development of revolutionary, new methods. We have specifically excluded descriptions of TE of teeth and special sensory organs, because these subjects have been covered elsewhere in this textbook.

Special challenges in oral and maxillofacial reconstruction

Virtually, all tissue types of ectodermal, mesodermal, and endodermal origin are candidates for TE strategies and are present in the oral and maxillofacial region. However, certain structures are more commonly impacted by disease, trauma, and developmental failures and constitute the focus of our discussion, though the reconstructive methods described can be applied to more rare conditions. Common *pathological* entities include both benign and malignant cystic and neoplastic processes affecting the upper (maxilla) and lower (mandible) jaws as well as degenerative conditions involving the mandibular articulation [temporomandibular joints (TMJs)] (Pictures 65.1–65.4). These diseases, or the subsequent removal of pathologically involved tissue, can produce continuity defects of the jaws requiring the replacement of bone, cartilage, and lining epithelium. Since many of these conditions are frequently silent and the dimensions of the structures involved relatively small, their initial presentation is usually associated with significant tissue involvement. In addition to disease, nonphysiological loading of bone can also produce loss of skeletal tissue affecting the jaws and joints. *Edentulous bone loss* involves the resorption of the alveolar processes of the jaws (i.e., that portion

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of the jaw bone surrounding the tooth roots) following tooth removal (Fig. 65.2). This phenomenon is believed to be the result of direct loading of bone during mastication and the loss of physiological maintenance forces transmitted by the teeth. Over time the loss of bone produces severely atrophied alveolar ridges, posing significant challenges to prosthetic reconstruction of the dentition and a predisposition to pathological fracture of the mandible [1].

Degenerative diseases of the TMJs are commonly the result of nonphysiological mechanical forces produced by excessive ranges of motion of the joints or chronic microtrauma from various parafunctional habits [2]. The habits in question include jaw clenching or tooth grinding (nocturnal bruxism) and repetitive motion habits such as nail biting and gum chewing. Injury to the TMJs commonly affects both the articulating surfaces of the condylar head and glenoid fossa as well as the interpositional disk, producing a spectrum of disease from chondromalacia to severe osteoarthritis. As a synovial joint, the TMJs can also fall victim to various immune-mediated disease processes such as rheumatoid or psoriatic arthritis. The inflammatory component is responsible for progressive structural tissue loss leading to changes in skeletal

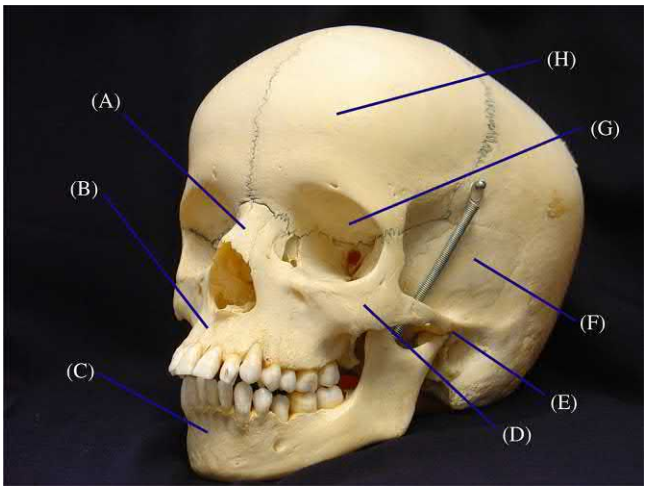
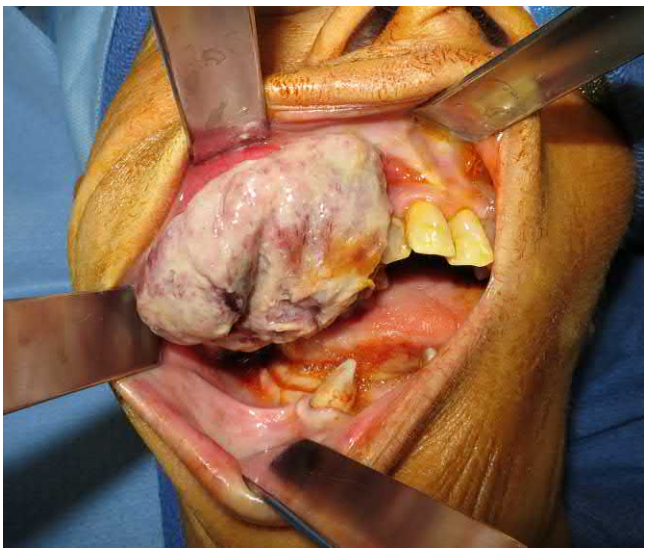
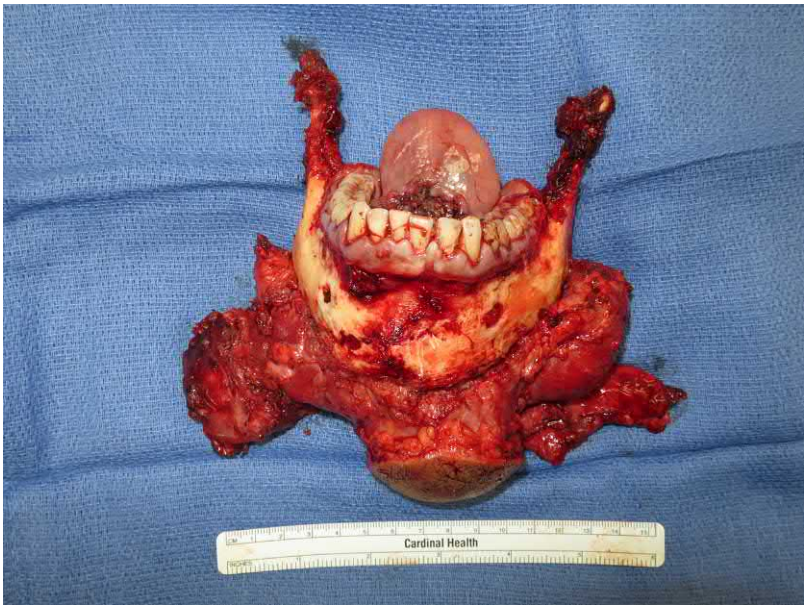


FIGURE 65.1 The human skull is a complex region composed of many bones. Several key structures include (A) nasal bone, (B) maxilla, (C) mandible, (D) zygomatic bone, (E) temporomandibular joint, (F) temporal bone, (G) orbital cavity, and (H) frontal bone.

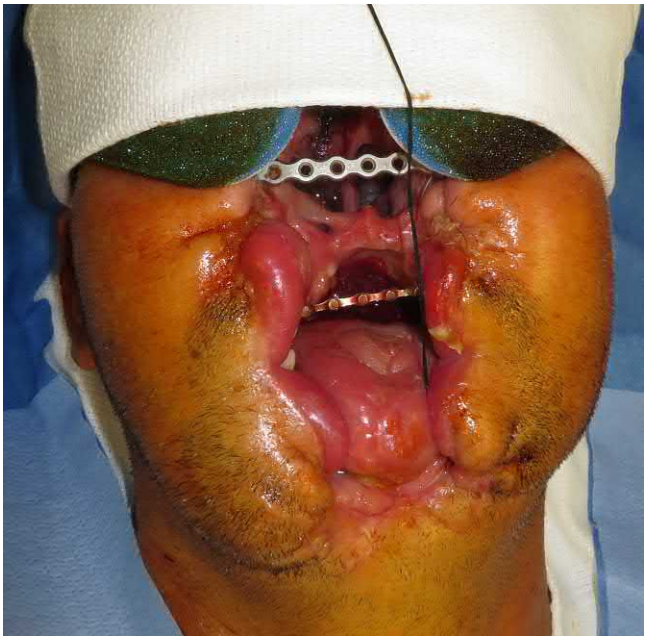


PICTURE 65.2 Osteosarcoma of the maxilla requiring resection with 3 cm margins.

PICTURE 65.1 Resection of ameloblastoma of the mandible next to a stereolithic model of tumor.



PICTURE 65.3 Squamous cell carcinoma of the floor of the mouth and tongue requiring composite resection of involved structures.



PICTURE 65.4 Self-inflicted gunshot wound with avulsive injury to both the maxilla and mandible.

relationships and malocclusion [3]. In advanced forms of disease, replacement of both cartilage and bone as a total joint reconstruction maybe necessary to restore function or skeletal support to the mandible.

Maxillofacial trauma constitutes another group of conditions providing opportunities for TE reconstruction. Whereas most forms of blunt trauma result in fractures where tissue loss is minimal, penetrating injuries produced by high velocity missiles and projectiles often create significant loss of bone and overlying soft tissue (Fig. 65.3). Finally, consideration should be given to the various forms of congenital facial clefts that commonly affect the oral and maxillofacial region. In a limited form, failure of the maxillary processes to fuse unilaterally or bilaterally produces alveolar clefts (Fig. 65.4). When the upper lip, maxilla, and palate are involved, a constellation of deformities associated with unilateral or bilateral cleft lip and palate patients is present.

In the reconstruction of anatomical defects, the causative events must be taken into account to ensure long-term success. Defects produced by traumatic, developmental, and pathological conditions are associated with a defined end point. Assuming that pathology has been completely eradicated or further traumatic insults do not occur, defects produced by these mechanisms can be fully characterized with respect to size and missing tissue types. In contrast, tissue loss as a result of parafunctional habits, nonphysiological loading patterns, and immunologically mediated degeneration often continues following reconstruction. This set of circumstances will adversely affect any biological constructs produced by TE techniques and impose an important limitation on the clinical application of their usage. Before biological, rather than alloplastic materials can be employed, correction of the underlying etiology is of paramount importance.

A special concern in oral and maxillofacial reconstruction is the potential exposure of grafted tissue to the external environment. Constructs used to restore defects involving the jaws, orbits, nose, and ears are potentially in direct contact with the mouth, sinuses (maxillary, ethmoidal, and frontal), nasal passages, and external environment (Picture 65.5). These areas are characterized by high

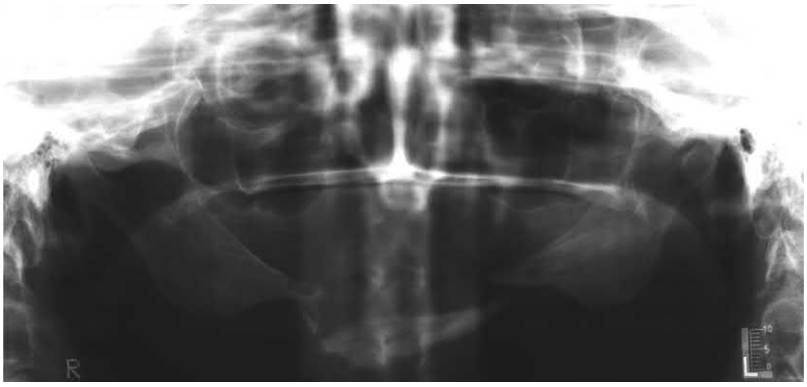


FIGURE 65.2 Panoramic radiograph of a patient with a fractured atrophic mandible. *Courtesy of Kamal Busaidy, D.D.S.*

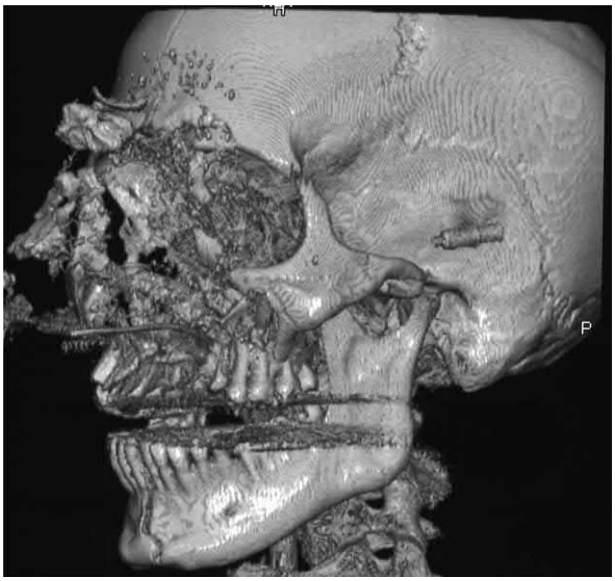


FIGURE 65.3 3D reconstructed radiograph of a patient with a self-inflicted gunshot wound demonstrating the significant disruption and loss of maxillofacial skeletal structures. *3D*, Three-dimensional.

moisture content, significant bacterial populations, and functional loads imposed by physiological activities such as chewing. If biological (i.e., tissue engineered) constructs are to survive under these conditions, modifications to account for the dilutional effects of moisture, presence of infective organisms, and mechanical loads must be provided by the engineered tissue. For example, when in vivo polymerization of materials is intended, the presence of fluid must be considered. Alternatively, preformed constructs can be used. Colonization of constructs with a mixed population of aerobic and anaerobic bacteria is expected with reconstructions involving oral, nasal, and sinus-related structures. Porous constructs, capable of harboring potentially pathological organisms, might be modified to reduce either bacterial attachment or replication until lining tissue develops over the implant forming a barrier to the external environment. In addition to contaminated wound sites, tissue constructs may be exposed to complicated mechanical loads before anisotropy is restored with the regeneration of biological tissue. Both the mandible and TMJs are subject to a combination of compressive, shear, and tensile loads depending on the type and degree of function [4,5].

Another special feature of the maxillofacial region is the number of tissue types within a relatively small region. As a result of this proximity, traumatic, pathological, and developmental events often lead to the creation of composite defects requiring reconstruction of multiple tissue types. This results in a special challenge not only to engineer composite tissues but also to attach the various constructs to each other in their normal anatomical relationship.

Facial symmetry is an important consideration in oral and maxillofacial reconstruction. Since most structures are paired or contiguous (e.g., the orbits, zygomas, and left and right maxillae and mandible), accurate reproduction of the external form is an important aspect to preserve facial esthetics. The paucity of overlying soft tissue as camouflage contributes to the exacting nature of oral and maxillofacial reconstruction and these requirements impose upon TE methods the ability to compose and maintain accurate morphology. The advent of new three-dimensional (3D) imaging techniques with the capacity to produce stereolithographic skeletal models that mirror both the normal anatomy and defect is a valuable adjunctive tool (Fig. 65.5). These models assist in the fabrication of scaffolds to support the reconstruction of missing tissue.

Current methods of oral and maxillofacial reconstruction

There are several methods used for oral and maxillofacial reconstruction and the selection of a particular modality takes into account a number of important issues. Major factors that guide this process include the presence (or absence) of associated soft tissue, the vascularity and vascular pattern present, a multidimensional characterization

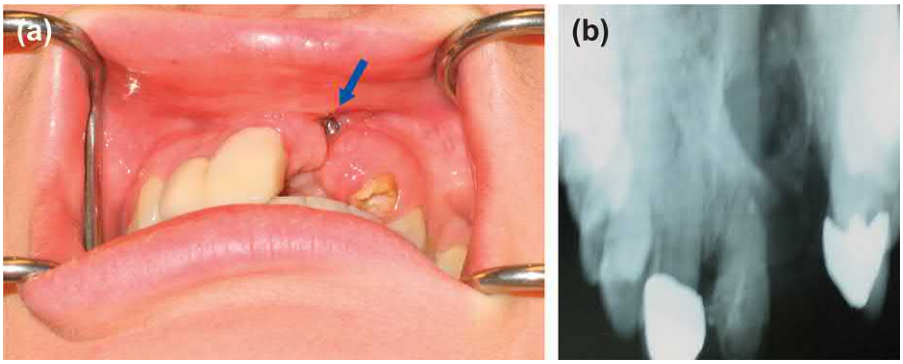
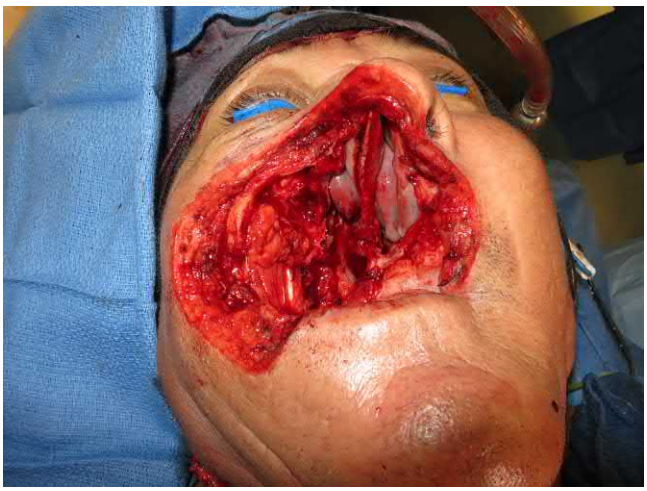


FIGURE 65.4 (A): Intraoral view of a left maxillary alveolar cleft. An oronasal fistula is present at the superior-most aspect of the cleft (see *arrow*). (B): Radiograph of a cleft of the maxillary alveolar bone.



PICTURE 65.5 Ablative defect of maxillary squamous cell carcinoma involving large defect of the midface.

of the defect size, the types of missing tissue, availability of tissue for transfer, and both patient and surgeon preference. Most reconstructive techniques can be categorized into four categories:

1. soft tissue pedicled flaps;
2. nonvascularized soft and hard tissue grafts (the graft establishes in a delayed fashion, a vascular network following implantation, relying upon tissue diffusion to preserve the viability of the transplant);
3. soft and hard tissue vascularized grafts (the graft is immediately perfused through an existing arterial-venous system); and
4. alloplastic reconstructions with prosthetic appliances.

On occasion, composite techniques can be used, such as the staged reconstruction of a defect where soft tissue is first added to a defect site followed by bone at a later time.

A newer technique using a hybrid of vascularized soft and hard tissue with nonvascular tissue engineered allogenic grafts has been reported in a limited number of cases but has shown promising results [6].

Mandibular defects

Reconstruction of the lower jaw is indicated following removal of tissue during surgical excision of a pathological lesion or following loss of tissue from a traumatic injury. When malignant disease is present, not only is more radical removal of tissue required but postoperative radiation therapy produces lasting compromise to both the cellularity and vascularity of the remaining tissue. Blast effects from missile injuries can also produce significant composite injuries with loss of bone soft tissue and diminished vascularity of the tissue bed. Two techniques are commonly employed for the reconstruction of mandibular defects. Vascularized grafts are indicated when the vascularity of the tissue bed is compromised by radiation or excessive scarring. They are also valuable when there is a requirement to replace both hard and soft tissue at the same time [7]. Hard and soft tissue defects can also be reconstructed using nonvascularized grafts, but their success relies upon an adequately vascularized tissue bed, which can be compromised in irradiated or traumatically injured tissue, to support the survival of transplanted cells before a new supply is established [8] (Picture 65.6).

As composite structures, vascularized grafts contain either soft tissue alone (muscle, subcutaneous tissue with or without epithelium) or include hard and soft tissue components (bone and soft tissue). Since the vascular supply to bone is contained within a peri-osseous cuff of muscle and fibrous tissue, it is not possible to transplant only bone. The additional tissue transferred into the site of a bony defect often produces a bulky graft. While this can be easily excised once a new vascular network is established, a second procedure performed several months after the initial transplantation is required. Another potential limitation to the use of vascularized bone grafts for the reconstruction of mandibular defects is the amount of bone available, since the dimensions of the graft are determined by the morphology of the donor site and not the size of the defect. Special techniques such as osteotomizing the graft and folding it upon itself have been described, but this can compromise the blood supply to

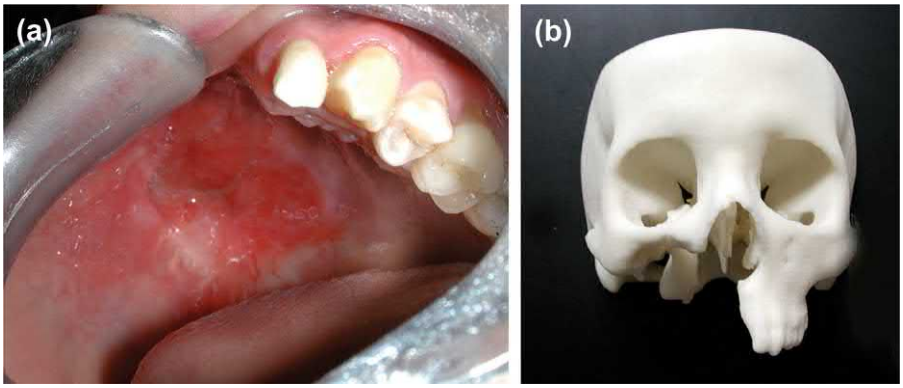
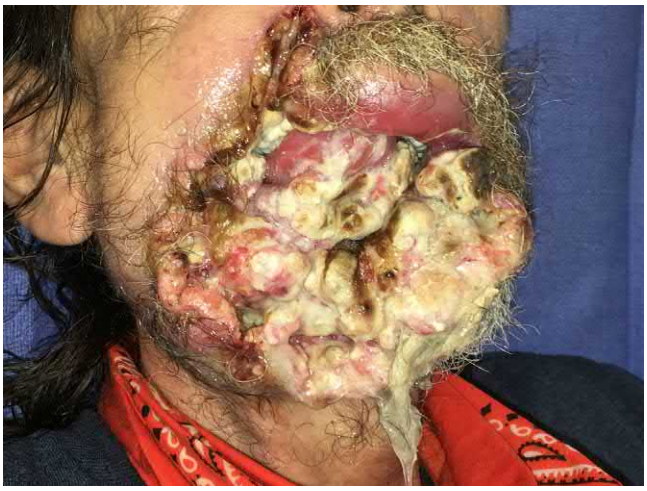


FIGURE 65.5 (A) Patient following a right maxillectomy for removal of a benign odontogenic neoplasm. Defect has filled in with fibrous tissue stimulated by grafting the site with an allogeneic dermal matrix. (B): Stereolithographic model of the same patient demonstrating the extent of the maxillary hard tissue defect.

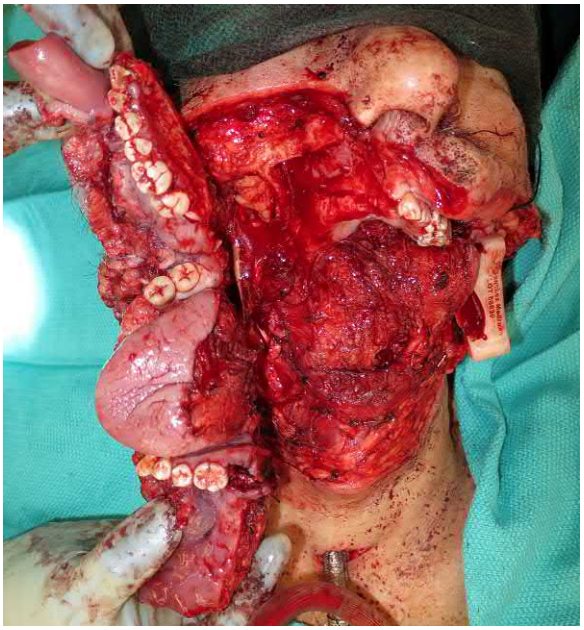
PICTURE 65.6 Placement of avascular bone graft in an infection-free bed via trans-cervical neck incision.



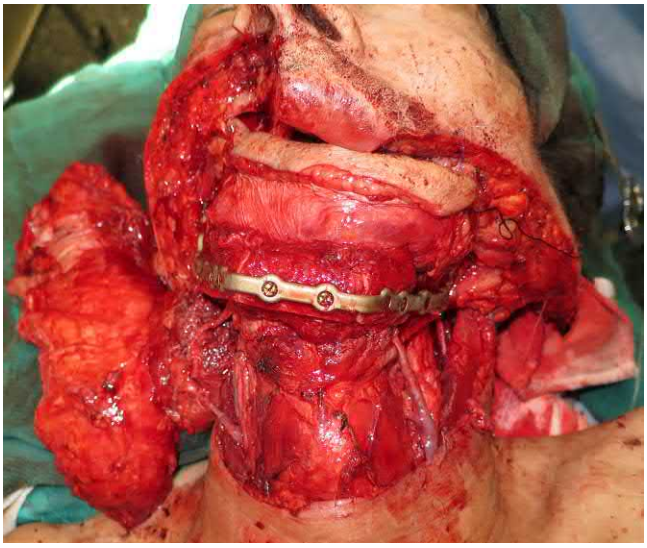
PICTURE 65.7 Massive squamous cell carcinoma of the lower lip.

the graft. Vascularized grafts are harvested from a limited number of anatomical sites characterized by a dominant arterial supply—venous drainage system. In addition, the en bloc harvesting of the graft must not compromise either the function of the donor site or the vascular and neural supply of structures distal to the harvest. Commonly used donor sites that meet these requirements include the fibula, ilium, scapula, and distal radius. Vascularized grafts transplanted to mandibular defects are anastomosed to patent vessels adjacent to the mandible, such as the facial, lingual or superior thyroid arteries, and veins. This reconstructive approach is highly technique-sensitive and while experienced microvascular surgeons achieve successful outcomes in over 90% of cases, less experienced surgeons or patients with underlying vascular disease (e.g., diabetes) enjoy less success (Pictures 65.7–65.10).

Mandibular defects can also be reconstructed using nonvascularized transplantations of autologous bone from various sites. Successful bone grafts rely upon adequate cellularity and a sufficiently cellular and vascular recipient bed. When the soft tissue bed is deficient or lacks a decent blood supply, an addition of well-vascularized soft tissue is achieved by the rotation of a muscle flap (with or without skin) into the mandibular defect. The pectoralis major, latissimus dorsi, and delto-pectoral flaps have all been described for this purpose. The bony reconstruction is delayed for a period of 3–6 months until the soft tissue flap has healed. In patients, whose soft tissue is adequate but avascular as a result of radiation therapy, hyperbaric oxygen therapy can improve the quality of the vascular supply in a course of treatments lasting between 4 and 6 weeks, where repeated exposures to pressurized room air promote tissue angiogenesis. This process adds both time and considerable expense to the reconstructive process but has been shown to be effective in improving the quality of the recipient bed. Once the soft tissue in a mandibular defect has been optimized with respect to quantity, cellularity, and vascularity, autologous bone is transferred from a donor site and molded to fit the dimensions of the defect. The bone graft can be retained with screws fixed to a rigid bone plate or held in position with the aid of cribs fashioned

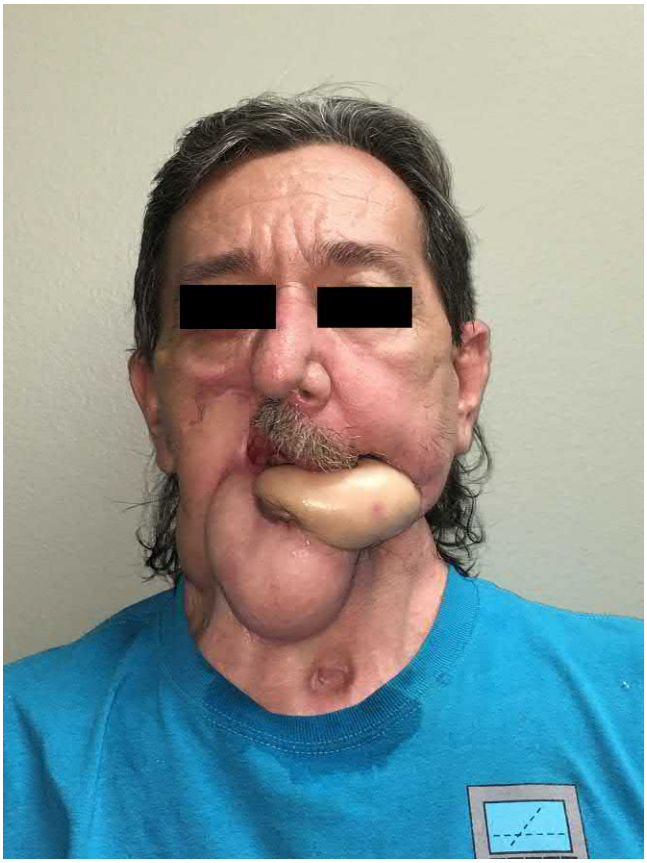


PICTURE 65.8 Resection involving lower mandible, including right midface and nose.



PICTURE 65.9 Use of anterior lateral thigh fasciocutaneous and fibula osteocutaneous vascularized free flaps for mandibular reconstruction.

either from processed allogeneic bone or alloplastic materials. Depending upon the size of the defect, bone can be harvested from the anterior ilium (suitable for defects up to 4 cm in length), the posterior ilium (defects up to 8 cm in length), and the tibia or mandibular symphysis and rami (defects of less than 2 cm in length). Nonvascularized grafts, especially those combined with allogeneic bone, are susceptible to infection especially following exposure to the intraoral environment, and in one case series, this was associated with a failure rate of

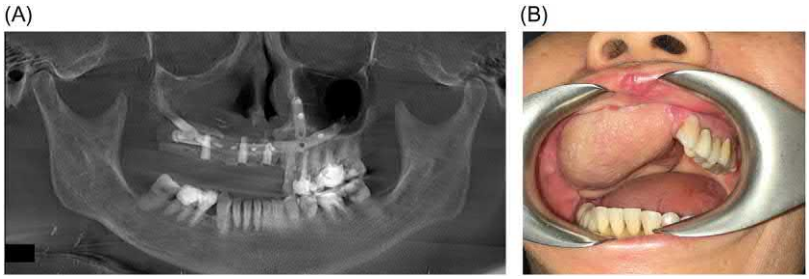


PICTURE 65.10 Patient 3 years postsurgery with no evidence of disease.

43% [9]. When a nonvascularized graft is colonized by organisms, infection often ensues and the graft fails to survive or integrate with the host bone. Aside from the potential for infection, nonvascularized grafts are less technique sensitive, allow "complete" reconstruction of a defect by customizing the volume of bone harvested, and are associated with less donor site morbidity [10].

Maxillary defects

Defects of the upper jaw pose difficult reconstructive challenges from several perspectives, but must be undertaken to preserve speech, prevent the escape of food and fluids during eating, and maintain esthetics. Unlike the mandible, which is related to the oral cavity alone, the maxilla is bounded inferiorly by the mouth and superiorly by the nasal cavity and maxillary sinuses. Even when present, the thin lining epithelium does not provide a sufficient cellular or vascular bed to support the transplantation of sufficient quantities of nonvascularized bone and the potential for exposure of the graft to oral and nasal environments is high. Postoperative or posttraumatic scarring reduces the tissue envelope even more, further



PICTURE 65.11 (A and B) The use of fibula osteocutaneous vascularized free flap for reconstruction of maxilla. Panoramic radiograph of reconstruction showing fibula, reconstruction plate, and dental implants (A). Intraoral postoperative photograph of the same patient showing bulk of soft tissue associated with flap (B).

complicating reconstructive efforts. Staged reconstructions have been described involving the initial transfer of vascularized soft tissue with a pedicled flap, such as the temporalis muscle or temporoparietal flap, followed by the addition of bone several months later [10]. As an alternative, vascularized flaps have been used because of their ability to transfer both hard and soft tissue at the same time [11]. However, the accompanying soft tissue and vascular pedicle may not be accommodated by the smaller dimensions of a maxillary defect and this has limited their use to hemi- or total maxillary reconstructions. The simultaneous transfer of a large bulk of overlying soft tissue also results in a postoperative recovery period of several months, where the flap can prevent mouth closure and compromise eating (Picture 65.11A and B).

As a result of these challenges, prosthetic appliances have become the most commonly used method to reconstruct maxillary defects. These devices incorporate teeth and a fitted base to separate the mouth from the superior defect [12]. Excellent restoration of both esthetics and function can be achieved, but the fact that they are not permanently fixed in place and, in fact, require daily removal and cleaning, reduces their acceptability by patients. In addition, adjustments are required periodically to account for remodeling of the underlying tissue bed.

The reconstruction of maxillary alveolar clefts is one exception to the use of prosthetic appliances as a primary reconstruction technique, even though they can be used very effectively to restore missing teeth in the cleft site or obturate an oral–nasal communication. When teeth are present in a cleft site, provision of bone is essential for eruption and support [13]. Alveolar grafting is therefore timed according to the presence and stage of development of adjacent teeth and is usually performed between the ages of 8 and 11 years. The procedure involves the development of soft tissue flaps to isolate the mouth from the nasal cavity and placing autogenous bone between the cleft segments to restore maxillary continuity. Loss of the graft from infection, insufficient vascularity, or lack of functional stimulus are not infrequent occurrences and opportunities for TE alternatives exist. This would be especially true if new interventions minimized the extent of surgery, since postsurgical scarring has been associated with restricted growth and development of the maxilla.

Relevant strategies in oral and maxillofacial tissue engineering

Driven by the limited supply and inherent shortcomings of various autogenous, allogeneic, and prosthetic materials currently used for the reconstruction of oral and maxillofacial tissues, the potential for tissue-engineered biomaterials as alternatives is under serious investigation with the hope that significantly improved therapies will result.

While a diverse number of strategies are presently under development, the fundamental tenets of TE remain the same. These include consideration of the biological and mechanical properties of the scaffold material and its interactions with relevant bioactive molecules and cell populations.

Although multiple tissue types exist in the oral and maxillofacial region, TE research in this field has focused primarily on the regeneration of single tissues: the bony craniofacial skeleton, lining epithelium, the cartilages of the TMJ, auricle, and nose, and the teeth and surrounding periodontal tissue (please refer to Chapter 69, Cutaneous epithelial stem cells and Chapter 71, Bioengineered skin constructs for teeth and periodontal regenerative initiatives, respectively).

Bone applications

An ideal biodegradable TE bone construct should combine the biocompatibility and osteoinductive potential of autologous bone, with the availability and structural characteristics of allogeneic bone. Additional scaffold design considerations include porosity, pore interconnectivity, surface chemistry, and the ability to reproduce complex 3D defects.

Scaffolds are responsible for a construct's initial mechanical integrity and provide surface area for cell attachment. Several biocompatible scaffold materials are currently used in oral and maxillofacial surgery, including naturally derived materials such as collagen, gelatin, or hyaluronic acid, synthetic polymers such as poly(lactic acid) (PLA) and poly(glycolic acid) (PGA), and ceramics such as calcium phosphate granules, blocks, and cements [14]. In addition to those materials clinically used, several

materials have been tested using in vivo models for craniofacial bone, including alginate, chitosan, poly(propylene fumarate), oligo(poly(ethylene glycol)-*co*-fumarate), poly(caprolactone), and polyurethanes [14]. These materials are typically processed as porous structures or hydrogels that guide the morphology of regenerated tissue, allow for tissue in-growth, and control the release of bioactive molecules such as growth factors or nucleic acids.

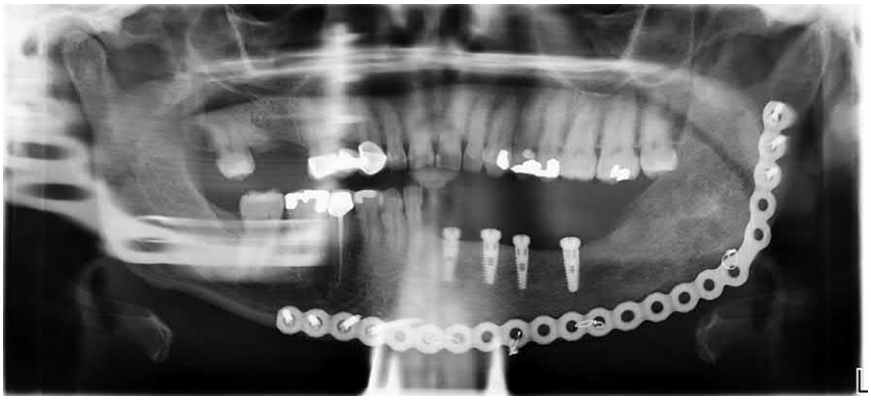
Other approaches to TE use novel biomaterials capable of implantation through minimally invasive surgery. This can be achieved via in situ cross-linking or polymerization via chemical reactions initiated by mixing chemicals immediately before injection, transcutaneous photopolymerization, or thermogelation [15–19]. Delivery of osteogenic factors or cells has been demonstrated with these techniques; however, parameters such as cell viability must be weighed against gel stiffness with such materials.

Growth factors act as mediators of cellular growth and differentiation during tissue regeneration and play an important role in extracellular matrix synthesis. Used as recombinant proteins in TE strategies, growth factors require a local population of target cells capable of affecting the desired response [20]. This constituency of cells maybe naturally present at the wound site or added to the scaffold at the time of fabrication [21] prior to implantation.

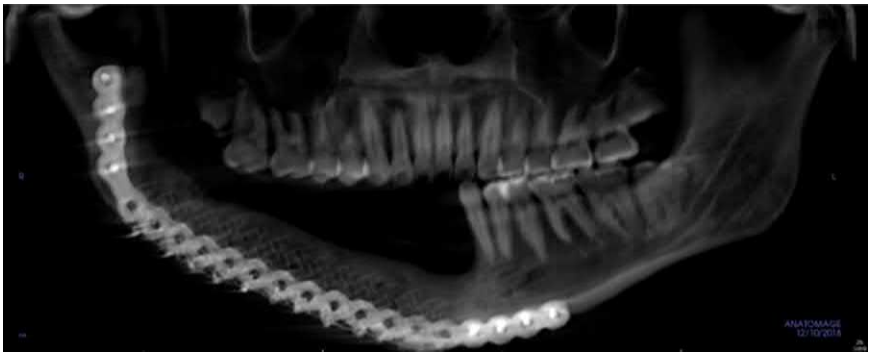
Factors that have been used for the regeneration of in vivo TE craniofacial bone include the bone morphogenetic proteins (BMPs) [21,22], transforming growth factor-beta (TGF-β) [23], fibroblast growth factors (FGFs) [24], insulin-like growth factors (IGFs) [25], and platelet-derived growth factor (PDGF) [26]. The bulk of experience concerning the use of growth factors for bone repair has involved BMPs [27] and this popularity has been extended into clinical investigations using recombinant human BMP-2 (rhBMP-2) for alveolar ridge augmentation [28,29], maxillary sinus floor augmentation [30,31], mandibular reconstruction following tumor resection [32,33], distraction-assisted alveolar cleft repair [34], and the treatment of medication-related osteonecrosis of the jaw [35,36]. A review by Herford et al. covers several cases of BMPs used in mandibular reconstruction secondary to tumor excision, trauma, and infection, highlighting the versatility of growth factor–mediated bone regeneration [37]. However, the review notes a relatively small number, 37, of documented clinical cases in the literature with a significant failure rate of 13.5%. This highlights the need for further investigation of these growth factor–based technologies in the craniofacial complex.

BMP-2 has found some definitive success in other aspects of oral and maxillofacial surgery, such as dental implants and sinus floor augmentation. Jung et al. [28] examined the effect of combining rhBMP-2 with a xenogeneic bone substitute in order to improve membrane-guided bone regeneration therapy of osseous defects in the areas of dental implant placement. Although there was not a statistically significant difference in percentage of newly formed bone at the rhBMP-2-treated site versus the control site at 6 months, a larger fraction of mature lamellar bone (76% vs 56%) was present in the experimental sites, as well as increased graft to bone contact (57% vs 29.5%). In addition, Boyne et al. [30] completed a phase II–randomized controlled study investigating the safety and efficacy of rhBMP-2 combined with an absorbable collagen sponge (ACS) versus bone graft for staged maxillary sinus floor augmentation. It was concluded that rhBMP-2 had a similar safety profile to bone graft with the added benefit of lacking donor site morbidity. In addition, the rhBMP-2/ACS treatment induced similar amounts of bone to the bone graft group, allowing for the placement and long-term functional loading of dental implants in approximately 75%–80% of the patients treated. More recently, Kim et al. [31] reported on a randomized controlled trial of 127 patients undergoing maxillary sinus floor augmentation. The safety and efficacy of rhBMP-2 mixed with hydroxyapatite granules was compared to bovine bone xenograft. Similar to previous work, new bone formation in the rhBMP-2-treated group was noninferior to that seen in the xenograft-treated group in the early stages after sinus augmentation with minimal side effects.

The clinical use of rhBMP-2 to regenerate much larger bone defects has also been reported in the literature. Melville et al. [32] described a case series in which five patients with mandibular continuity defects underwent immediate transoral reconstruction using a combination of bone allograft, bone marrow aspirate concentrate, and rhBMP-2. Despite the small number of patients in the study, a success rate of 100% was observed with all patients showing excellent bone quality both clinically and radiographically for endosseous dental implant placement (Pictures 65.12 and 65.13). Carstens et al. [34] described the use of "distraction-assisted in situ osteogenesis" to treat a severe facial cleft, in which rhBMP-2/ACS implantation was combined with distraction osteogenesis to create the patient's ramus and condyle as part of the surgical reconstruction. A similar spectacular application of rhBMP-7 has been described by Warnke et al. [38] for the reconstruction of a 7 cm mandibular continuity defect in a patient who had received ablative tumor surgery and subsequent radiation treatment. A bone-muscle-flap prefabrication technique was used, in which computed tomography and computer-aided design techniques were used to fabricate a custom titanium mesh cage replicating the contours of the missing mandible. Within this cage a combination of xenogeneic bone mineral blocks coated with rhBMP-7 and autologous bone marrow were placed,



PICTURE 65.12 Panoramic radiograph demonstrating regeneration of hemi-mandible using tissue-engineering strategy (rhBMP-2 + bone marrow aspirate concentrate + allogeneic bone), 3 years postresection of ameloblastoma and reconstruction. *rhBMP-2*, Recombinant human BMP-2.

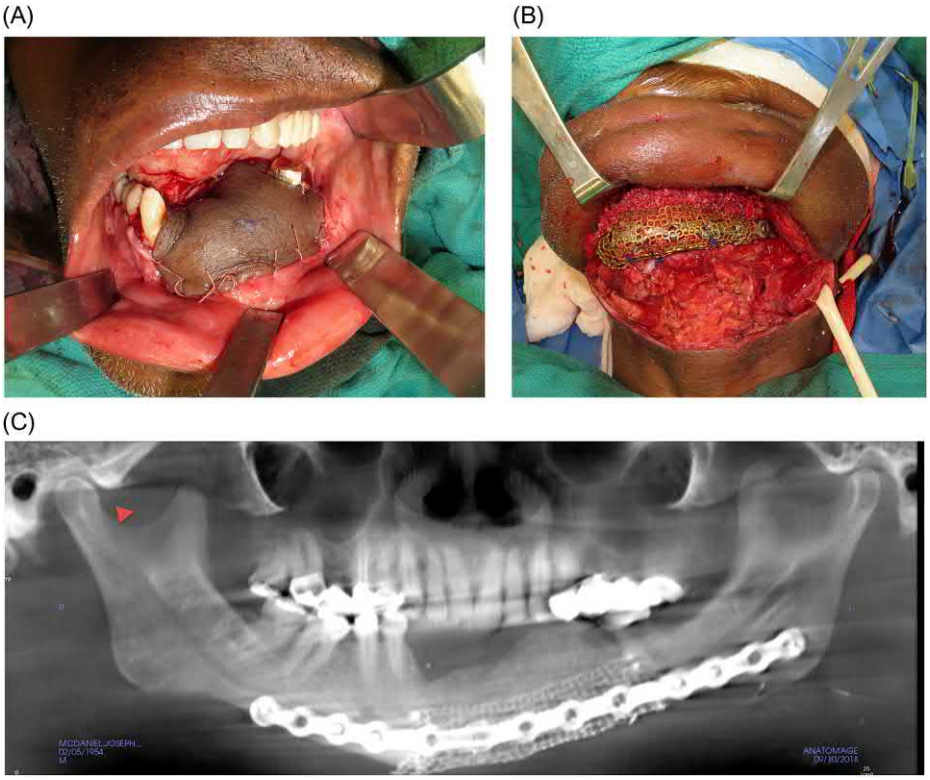


PICTURE 65.13 Panoramic radiograph of secondary-staged tissue-engineered graft (rhBMP-2 + bone marrow aspirate concentrate + allogeneic bone), 2 years following placement of graft. *rhBMP-2*, Recombinant human BMP-2.

prior to implantation of the entire construct within the latissimus dorsi muscle of the patient. Following 7 weeks of implantation within this "in vivo bioreactor," the viable mandibular replacement was harvested from the patient along with part of the muscle containing a major artery and vein which were subsequently anastomosed with vessels at the recipient site using microsurgical techniques. Four weeks after this transplantation surgery, the patient was able to undertake a small amount of mastication and enjoy more solid foods. In a similar case of prevascularization, Mesimäki et al. implanted a titanium mesh containing β-TCP granules seeded with adipose-derived stem cells (ASCs) cultured in rhBMP-2 into the rectus abdominis muscle of a patient who had undergone hemimaxillectomy [39]. The mesh was harvested with a vascular pedicle 8 months later and anastomosed to vasculature in the face replacing the resected bone. This patient went on to receive dental implants for complete dental rehabilitation. Others have reported the use of "hybrid" surgical approaches, in which a vascularized free flap is combined with autologous bone grafting [6] (Picture 65.14A–C).

A significant drawback to growth factor strategies in TE is the shortage of naturally derived factors isolated from biological tissue. This deficiency has been addressed with the development of techniques to produce biologically active proteins using recombinant engineering techniques. However, the use of recombinant proteins is not without concern [27]. Compared to animal models, bone regeneration in humans does not appear to be as robust. In order to overcome this species recalcitrance, administration of factors in excess of naturally occurring concentrations appears to be necessary. The augmented administration of exogenous factors may potentially stimulate harmful biological effects such as malignant transformation of cells and also prove to be too expensive when compared to alternative techniques for tissue regeneration. In an effort to mitigate cost, potential for harmful stimulation or disease transmission, autologous supplies of growth factors have been investigated, primarily through the use of platelet-rich plasma (PRP). As a natural source of growth factors, PRP has been applied to craniofacial bone TE scaffolds, but with limited success, illustrating the inherent difference in the effect of therapies across species [40,41].

Attempts to address the shortcomings of recombinant protein-based strategies have spurred investigation into the use of gene delivery for TE. By delivering the gene for the expression of a protein with specific effects on a target cell population, successfully transfected cells will elaborate the protein constitutively. This results in higher and more constant levels of protein production [42]. However, while both viral [43,44] and nonviral [45] gene



PICTURE 65.14 (A, B, and C) Hybrid combination of radial forearm osteocutaneous vascularized flap for intraoral soft tissue coverage (A) with rhBMP-2 + bone marrow aspirate concentrate + allogeneic bone graft (B). Panoramic radiograph showing excellent regeneration of bone 8 months out (C). Patient is ready for dental implants. *rhBMP-2*, Recombinant human BMP-2.

delivery vectors have been used for bone regeneration in cranial defect animal models, compromises must be made with each. Adenoviral constructs have commonly been used as viral vectors to transfect craniofacial tissues and have the advantage of efficiently transfecting both replicating and quiescent cells [46]. In addition, adenoviruses are easily manipulated, can be produced in high titers, and large amounts of genetic information can be inserted into them. However, concerns related to viral vectors include in vivo homologous recombination and the possibility of an immune response from the expression of viral antigens on the surfaces of transfected cells. These concerns have led to the development of nonviral vector agents [42].

While numerous nonviral gene delivery systems exist, a common problem is their low in vivo transfer efficiency [46]. Nonetheless, such systems are able to deliver much larger genes with minimal immunogenicity. One promising modality of nonviral gene delivery for craniofacial applications is the use of cationic liposomes which have been used to regenerate cranial bone defects in rabbits by delivering BMP-2 plasmid cDNA [47]. The low transfection efficiency of uncondensed, naked plasmid DNA has also been addressed by the use of the cationic macromer poly(ethylene imine), which has been used to condense BMP-4 plasmid DNA and deliver it in a sustained and localized manner from poly(lactic-*co*-glycolic acid) scaffolds within critical size cranial defects [45].

Gene transfection can take place directly within the defect site by releasing the delivery vector in vivo from the TE scaffold [43,44]. Indirect delivery methods have also been described using a target cell population harvested from the patient, performing in vitro transfection of the cells, and then reimplanting the transfected cells into the defect along with the TE scaffold material [48]. While the direct technique may be simpler, it has a lower transfection efficiency and target cells in a nonspecific manner [27]. The indirect ex vivo approach, on the other hand, requires additional harvesting and culturing procedures but avoids the risks associated with placing viral vectors directly into the patient and disturbing the host genome. Ex vivo—transfected cells are not immunologically privileged and may still express viral antigens on their surface which can lead to a host response following implantation.

As a corollary to gold standard approaches where bone grafts and flaps include the donor site cells, some TE approaches to craniofacial reconstruction employ cell-seeded scaffolds as implants. These have potential benefits for regenerating tissues in large defects or those with compromised healing capacity, such as those affected by radiation therapy [8]. The majority of cell-seeded scaffolds have investigated mesenchymal stem cells (MSCs) or ASCs. Reviews have covered some of the works in these areas looking at various stem cell sources, delivery, and other parameters such as in vitro expansion and

differentiation [8,49]. To highlight a few studies, MSCs were applied to ceramic and polymer scaffolds with and without PRP in both cranial and alveolar defects of rats and minipigs, respectively [40,41]. In both studies the addition of MSCs enhanced bone regeneration over all other groups irrespective of PRP presence. In another study, autologous, culture-expanded MSCs were used in combination with alginate hydrogels for the treatment of large cranial bone defects in sheep [50]. Finally, predifferentiated ASCs were applied to rabbit cranial defects on gelatin scaffolds showing enhanced bone regeneration within the defect [51]. However, in another rabbit cranial defect study, rhBMP-2 on collagen regenerated greater amounts of bone compared to ASCs or predifferentiated ASCs, indicating that although cell delivery for bone regeneration in the craniofacial complex shows promise, issues of cell sourcing, purification, and processing need to be investigated further [52].

Aside from the biological components of TE constructs, scaffold properties are also extremely important to the overall success of any particular strategy. A common misconception is that bone TE scaffolds for craniofacial applications do not require substantial strength, since the craniofacial skeleton is not subjected to heavy loading. However, in vivo studies demonstrate that many craniofacial bones undergo levels of strain similar to that experienced by the appendicular skeleton [53], substantiating the need for mechanical strength of potential bone TE scaffolds. Ideal scaffold design must therefore reconcile the need for high porosity and interconnectivity, which promotes tissue in-growth and scaffold degradability, with a requirement for mechanical strength. Computational methods for designing and fabricating scaffold architectures to optimize both pore interconnectivity and load bearing characteristics have been performed [54], and a proof of concept study has illustrated the effectiveness of scaffold design in fabricating a mandibular condyle for a minipig [55].

The surface characteristics of bone TE scaffolds also determine their ability to regenerate tissue in the wound-healing environment. Surface chemistry has a significant effect on the interactions between the cell populations present in the defect and the biomaterial. Hydrophilic synthetic polymers such as oligo(poly(ethylene glycol) fumarate) (OPF) have been shown to impede bone healing in extraction sockets as compared to the hydrophobic polymer poly(propylene fumarate), based on the OPF macromer's prevention of protein adsorption and hence cell adhesion [56]. Interestingly, the resistance of OPF hydrogels to generalized cell adhesion has been used to advantage in the fabrication of biomimetic scaffolds, which are able to selectively encourage the migration of osteoblasts in vitro through the addition of specific binding peptides to their surfaces such as osteopontin-derived peptide [57].

In addition to surface chemistry, surface topography can impact healing. In a series of studies, porous materials in the mandibles of New Zealand White rabbits were shown to enhance the healing of the overlying mucosal surface in a composite tissue defect model [58–60]. However, contrary to traditional TE materials with high porosity led to an increased inflammatory response not seen with lower porosity materials [58]. Two of these studies investigated porous poly(methyl methacrylate) (PMMA), a material currently part of many Food and Drug Administration–cleared products. By creating porous PMMA and enhancing soft tissue interaction with the implant, these materials show promise for rapid translation to clinical use in a staged approach, whereby the implant serves to temporarily maintain the bone space during soft tissue regeneration and is removed for the definitive therapy [61].

Cartilage applications

While there has been extensive research in bone TE in the craniofacial complex, there has been less research in cartilage TE. However, cartilage TE in the craniofacial complex remains a significant challenge due to the inherent lack of remodeling in cartilage due to decreased cellularity and vascularity and due to the various cartilaginous tissue types in the craniofacial complex. As in musculoskeletal TE, articulating cartilage is part of the TMJ on the mandibular condyle, while the TMJ disk has different morphology. In addition, there is structural cartilage in the nose and ears, which supports the skin to create openings critical for the senses.

The earliest TE study directed at reconstruction of the TMJ disk used a porous collagen scaffold seeded with articular cartilage cells. After 2 weeks the construct appeared similar to a disk with regards to gross morphology and cell shape [62]. Later efforts tested fibers of PGA and PLA and concluded that both materials were able to support cell attachment and matrix production and exhibited acceptable mechanical properties after 12 weeks [63]. Another study comparing PGA, polyamide filaments, expanded polytetrafluoroethylene filaments, and bone blocks [64] demonstrated cell attachment and limited collagen production, but neochondrogenesis was not observed after 4 and 8 weeks. While PGA is an acceptable scaffold substrate, the material degrades exceedingly rapidly, leaving constructs with limited mechanical integrity after only a few weeks. As an alternative, PLA nonwoven mesh has been tried and initial results show promise with retention of tensile and compressive integrity over a similar time scale [65]. However, replacement of fibrocartilage to restore the articulative surfaces or interpositional disk of the TMJ's constitutes a special challenge, including identifying a suitable source

of healthy fibrochondrocytes. In a review by Wang et al., cell sources for TMJ TE are discussed [66]. Primary cells such as chondrocytes from articular cartilage or the TMJ disk have enhanced phenotypic characteristics for TE but require expansion, which commonly results in dedifferentiation and loss of this phenotype [67,68]. To address this limitation, various protocols have been developed to reprogram expanded, primary cells back to a chondrogenic phenotype using a cocktail of growth factors and aggregate culture [69–72]. Stem cells, on the other hand, proliferate extensively and can be induced into a chondrocyte-like cell through growth factors and other parameters. In a study by Bailey et al., condylar chondrocytes were compared human umbilical cord cells on PGA scaffolds and were not only able to proliferate more but also produced more extracellular matrix with components similar to that of native articulating cartilage [73]. Beyond differences in cell type, chondrocytes from various anatomical regions have shown varied ability to proliferate or synthesize extracellular matrix components in vitro. Specifically, costal and ankle chondrocytes have shown greater synthesis of collagen and glycosaminoglycans (GAGs) compared to chondrocytes harvested from the TMJ disk [74,75]. Other studies have investigated paracrine and cell contact signaling by coculturing differentiated cells together or with undifferentiated cells [76–78]. In these studies, coculture of fibrochondrocytes with either chondrocytes or with embryonic stem cells resulted in greater extracellular matrix production.

In addition to cell types, growth factors have been used to enhance the regeneration of cartilage tissue for the craniofacial complex. This potential was first observed in an experiment comparing the effects of TGF-β_1 with prostaglandin E_2 (PGE_2) on bovine TMJ disk cells in monolayer. TGF-β_1 increased cell proliferation 2.5-fold, while PGE_2 had no significant effect [79]. The effects of PDGF, IGF, and basic FGF (bFGF) have also been assayed using monolayer cultures of porcine TMJ disk cells. The results of these studies suggest that lower concentrations favor biosynthesis, while higher concentrations favor proliferation [80]. The most beneficial growth factors appear to be IGF-I and bFGF, both of which produce significant increases in collagen synthesis and cell proliferation [75,80]. Since native tissue is exposed to a variety of growth factors, combination strategies are likely to prove more beneficial than single factor therapy. To explore this hypothesis, IGF-I and bFGF in low concentration were combined with bFGF and TGF-β_1. This cocktail successfully demonstrated increased collagen production when applied to porcine TMJ disk cells seeded on PGA scaffolds [81]. However, while constructs exposed to growth factor combinations improved structural integrity and overall cellularity, a statistically significant improvement in biochemical or mechanical properties was not demonstrated [82]. Finally, other combination strategies employed coculture with growth factor delivery, which were shown to increase matrix production and structural integrity of a gel construct over the individual strategies alone [76,77].

The TMJ is not the only cartilaginous tissue of the craniofacial complex, and research into cartilage TE of the ear has also been investigated. In vitro expansion and seeding of harvested chondrocytes into degradable polymer scaffolds is capable of producing cartilage following implantation in immunocompromised and immunocompetent animal models [83], but the clinical experience has been somewhat disappointing with resorption of tissue-engineered auricular cartilage after several months [84]. However, to enhance the structural aspects of engineered cartilage and to increase the viability and maintenance of overlying tissue, Lee et al. seeded porous polyethylene implants with chondrocytes in fibrin gels [85]. These implants showed dimensional stability unlike previous auricular tissue-engineered constructs.

Although growth factors have received the most attention, positive biochemical stimulation is also likely to come from culture conditions and cellular interactions, as well as matrix modifying enzymes. An ascorbic acid concentration of 25 μg/mL has been shown to produce constructs with higher total collagen content and higher aggregate modulus relative to concentrations of 0 or 50 μg/mL [86]. This was likely associated with improved seeding observed for the constructs cultured in 25 μg/mL of ascorbic acid. In addition, a concentration of 100 μg/mL of glucosamine sulfate was found to enhance condylar cell proliferation and matrix production as a media supplement [75]. Metabolic supplements have also been shown to impact cell proliferation and matrix synthesis. L-Glutamine, sodium pyruvate, and insulin increased cell proliferation, while L-proline at high concentrations decreased matrix production [87]. Initial cell seeding is another important consideration in any TE construct due to cell-to-cell interactions and signaling. It has been shown that PGA scaffolds seeded at saturation increased cellularity and extracellular matrix (ECM) content relative to scaffolds seeded below saturation [88]. Matrix modifying enzymes, such as chondroitinase ABC and lysyl oxidase–like 2, also have beneficial effects on constructs. When combined with TGF-β_1, these agents increased tensile Young's modulus and ultimate tensile strength in neofibrocartilage by 245% and 186% compared to controls, correlating with picrosirius red staining [89].

The native TMJ disk undergoes significant compression, tension, and shear [90]. While cells proliferate and produce ECM in static culture, mechanical stimuli maybe required to produce an optimal tissue–engineered construct. A variety of mechanical stimuli maybe beneficial, including compression, tension, hydrostatic pressure, and fluid shear

stress. Two extensive reviews of the mechanical bioreactors that have been used in engineering cartilaginous tissues have been published on the subject [91,92].

Several studies have investigated the effects of mechanical stimulation on TMJ disk constructs. A low-shear fluid environment by means of a rotating wall bioreactor created constructs with dense matrix and cell composition [93]; however, when the biochemical content of these constructs was compared to those grown in static culture, no clear benefit of the bioreactor was observed. When disk cells were exposed to hydrostatic pressure in monolayer or PGA scaffolds, constant hydrostatic pressure at 10 MPa increased collagen production compared to static culture [94]. In contrast, intermittent hydrostatic pressure from 0 to 10 MPa at 1 Hz frequency was detrimental to the constructs, producing less collagen and GAGs than unloaded controls. These results were consistent in both two-dimensional and 3D culture. In another recent study, dynamic tensile strain significantly reduced interleukin-1β-induced upregulation of matrix metalloproteinase (MMP) [95]. This may have implications on future TE studies, since MMPs play an important role in ECM degradation and remodeling. In a study examining tension for articular cartilage engineering, continuous tensile stimulation−coupled bioactive stimuli resulted in neocartilage with tensile properties 5.8-fold higher than untreated controls [96]. Using costal chondrocytes, passive axial compression has also been studied for articular cartilage engineering and has shown to increase compressive relaxation modulus with bioactive treatments when compared to just bioactive factors [97]. Models for other tissues, such as articular cartilage, might serve to further inform mechanical stimuli for the TMJ.

Until recently, TMJ disk TE was limited to the benchtop, and there was a small quantity of successful in vivo studies, particularly using large animal models. A recent orthotopic, in vivo study in the Yucatan minipig showed successful regeneration of the TMJ disk using allogeneic tissue−engineered constructs [98]. Implants were surgically inserted into the TMJ disk via the novel intralaminar fenestration surgical technique. Compared to empty defect controls, construct implantation resulted in 3.4-, 3.2-, and 4.4-fold higher tensile Young's modulus, integration, and defect closure. The study also cited a limited immune response via immunohistochemical stainings for macrophages, T cells, and B cells. This study demonstrates feasibility of regenerating the TMJ disk using TE, and studies such as these will continue to propel the field toward translation of TMJ cartilage TE.

Oral mucosa applications

Oral mucosa regeneration has followed many of the strategies of skin TE, in that cultured epithelial sheets showed inadequate results due to fragility, contractility, and failure due to lack of underlying supporting tissue. Thus many approaches have considered the use of thick cultured grafts containing single or multiple cell types. An extensive review by Moharamzadeh et al. covers scaffold materials, cell sources, and culture medium [99]. Many of the same materials have been investigated for oral mucosal engineering as in bone and cartilage such as collagen, fibrin, gelatin, PLGA, and PCL. In addition, various cell sources have been used, primarily keratinocytes and fibroblasts either from oral or skin origins. Finally, growth factors such as epidermal growth factor have been employed to promote proliferation. In one study, fibroblasts and keratinocytes were cultured on a collagen composite scaffold [100]. The culture led not only to cell-specific markers for a full thickness mucosa but structural components, including a basement membrane and extracellular matrix. A similar study used gingival fibroblasts and keratinocytes harvested from patients on a clinically available collagen matrix, resulting in cell markers and tissue structures similar to mucosal tissue [101]. In vivo, mucosal grafts of collagen precultured with gingival fibroblasts and keratinocytes have maintained their phenotype as mucosal tissue after 60 days of implantation [102]. Approaches which culture autologous oral keratinocytes and fibroblasts on a "scaffold" of fibrin obtained from autologous plasma have also been reported [103].

Several groups have shown translation of a tissue-engineered mucosal graft. Creating a mucosal graft in vitro using canine cells cultured on AlloDerm, the group translated this technique by first using human cells in vitro [104,105]. Subsequently, in a 30-patient study comparing AlloDerm alone to AlloDerm precultured with autologous cells, the precultured grafts show enhanced wound healing with earlier vascularization and maturation of the submucosal layer at 28 days after grafting [106]. Tissue-engineered oral mucosal grafts for urethral reconstruction have also been evaluated in a multicenter, prospective observational trial where they were shown to be safe and efficient in urethroplasty [107]. Lastly, Schmitt et al. [108] showed in a prospective clinical trial that the collagen matrix Mucograft could be used to regenerate peri-implant mucosa with sufficient long-term stability comparable to autologous free gingival grafts.

In addition to oral mucosa, composite grafts for mucocutaneous junctions such as the lips have been explored. Peramo et al. present a novel approach to creating these junctions, where oral mucosal cells and skin cells are cultured on AlloDerm in vitro with a separation barrier [109]. This barrier is lifted and the cells are allowed to migrate into the junction space and interact. The cell construct was then lifted to air−liquid interface for maturation of the construct and characterized for morphology and immunohistochemical staining for keratin content.

Biochemical markers were consistent with spatial distribution in mucocutaneous tissues, illustrating the possibility of this strategy to be employed for TE lips.

Composite tissue applications

Success with the regeneration of single tissue types such as bone and cartilage has encouraged investigators to attempt the reconstruction of structures composed of multiple tissue types. Such anatomic structures may exist as composites of hard and soft tissues, which differs in their cellular composition and mechanical properties, yet perform as a single functional unit [110].

The TMJ condyle serves as an example of a maxillofacial composite structure consisting of articular cartilage and subchondral bone and provides an excellent opportunity for composite osteochondral TE. A study performed by Alhadlaq et al. [111] used adult bone marrow MSCs, expanded in culture and induced to differentiate into separate osteogenic and chondrogenic lineages in vitro. The resultant cells were then encapsulated in poly(ethylene glycol)-based hydrogels and the cell−polymer solutions cross-linked in a mold which provided the correct stratified organization of the osteogenic and chondrogenic layers. Finally, the osteochondral constructs were implanted into the dorsum of immunodeficient mice for up to 8 weeks. Histological and immunohistological analyses revealed both structural and immunohistochemical differences between the osteogenic and chondrogenic layers, which served as a primitive proof of concept of the potential for composite tissue−engineered constructs in the craniofacial region. In a similar study, trying to regenerate tissue within an osteochondral defect, a gradient scaffold releasing BMP-2 on the osteogenic side of the scaffold and TGF-β_1 on the chondrogenic side was fabricated [112]. This construct showed increased osteo- and chondrogenesis on the respective sides.

Animal models

As TE strategies become more sophisticated, the complex interactions between multiple cell populations, growth factors, and scaffold biomaterials require testing within clinically relevant in vivo healing environments. While computational models and in vitro testing provide proof-of-concept verification of a particular TE strategy, more challenging test beds using animal models are ultimately required, to allow discrimination of the healing potential of different biomaterial constructs [113].

The ideal model for testing the validity of tissue-engineered bone constructs is the *critical size defect* (CSD): namely, an intraosseous defect which will not heal by bone formation during the lifetime of the animal [114]. Several CSD models have been described in the literature, including the calvarial defect [23,48] and long bone segmental defect [115,116]. These models do not accurately describe the oral and maxillofacial environment, because they do not simulate the unique masticatory stresses and cell populations seen in mandibular or maxillary wound healing. Mandibular defects have been described in rats, but they have the disadvantages of poor surgical access and a tendency for implanted materials to fall into the fascial spaces [114]. Conversely, larger animals such as dogs [117], minipigs [118], goats [119], and nonhuman primates [120] offer the advantages of easy surgical access and the ability to create large defects but are expensive to maintain. As such, a critical size mandibular defect in a rabbit was developed. This 10 mm, cylindrical, bicortical defect in the New Zealand White rabbit has been shown to be nonhealing after 16 weeks (Fig. 65.6) [121,122]. In addition, this model was

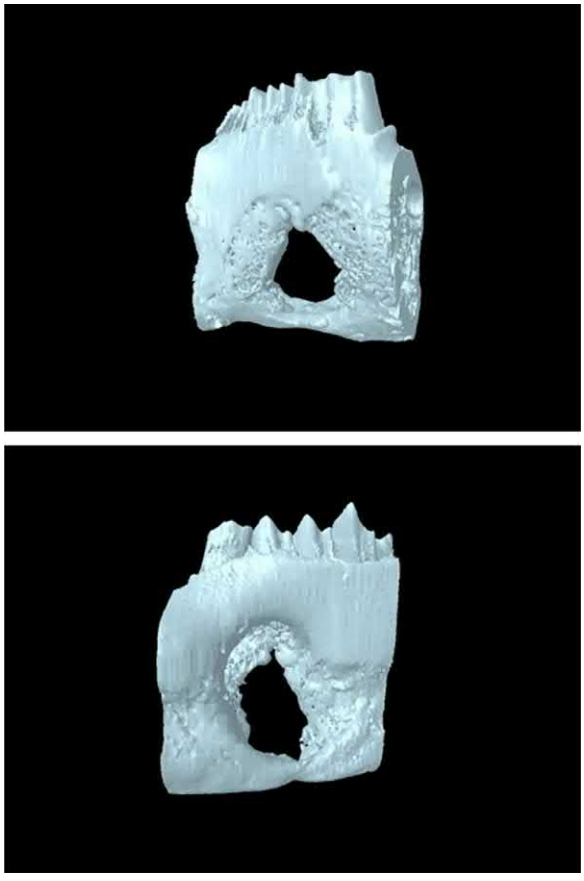


FIGURE 65.6 Micro-CT-generated representative three-dimensional reconstructions. Note the buccal aspect of the specimen is shown on the top, with the lingual aspect on the bottom. The critical size defect at 16 weeks shows a "through-and-though" defect and a lack of bony bridging across its center. *CT*, Computed tomography. *Reprinted from Young S, Bashoura AG, Borden T, Baggett LS, Jansen JA, Wong M, et al. Development and characterization of a rabbit alveolar bone nonhealing defect model. J Biomed Mater Res A 2008;86(1):182−94 with permission.*

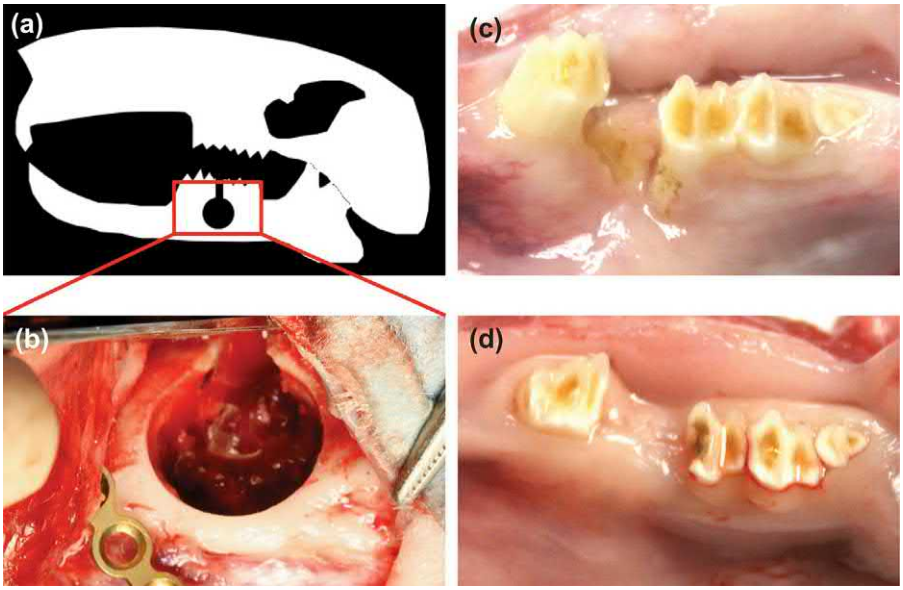


FIGURE 65.7 Schematic (A) and intraoperative photo (B) illustrating the composite tissue defect model of the rabbit mandible with a critical size bone defect and overlying mucosal defect. A healed (C) and nonhealed (D) mucosal defect are seen after 12 weeks of biomaterial implantation. *Reprinted from Spicer PP, Kretlow JD, Henslee AM, Shi M, Young S, Demian N, et al. In situ formation of porous space maintainers in a composite tissue defect. J Biomed Mater Res A 2012;100(4):827–33 with permission.*

expanded into a composite tissue defect model, whereby an overlying mucosal defect is created (Fig. 65.7) [58–60]. Composite tissue defect models represent an important direction for evaluation of tissue-engineered constructs. Defects resulting from trauma and resection of pathology frequently involve multiple tissue types and the combination of successful therapies for each individually may not suffice when both tissues need regeneration.

The future of oral and maxillofacial tissue engineering

While significant progress has been made toward our ability to fabricate tissue in the laboratory for the reconstruction of defects in the oral and maxillofacial skeleton, considerable challenges remain before these techniques are embraced as a feasible clinical modality. Some of the issues pertain to TE itself, such as the ability to identify and harvest a suitable population of cells capable of fulfilling the functions of the desired tissue, supporting cellular differentiation and reproduction through physiological levels of growth and attachment factors, promotion of vasculogenesis, and the development of matrices that fulfill the physical requirements of a skeletal site. Since many of the structures in the oral and maxillofacial region are composed of multiple tissue types, the ability to engineer composite structures is also important, as is consideration of the unique environment that tissue-engineered constructs are exposed to. Even as these demands are met, other factors must be considered before TE tissue can be adopted as part of a reconstructive surgeon's armamentarium. Existing reconstructive techniques were originally based on macroscopic concerns for restoring the shape and size of a missing structure. Since TE is essentially based upon cells, a microscopic appreciation of the function of a particular tissue type must first be derived. At this time, characterization of the function and pathological degradation of many of the structures in the oral and maxillofacial region is unfortunately deficient. In other words, before the replacement for a structure can be appropriately engineered, a better understanding of the function and local environment must first be achieved. This is particularly true of defects produced by on-going pathological processes where correction of the condition must precede replacement with yet another biological substrate. The future of oral and maxillofacial TE therefore lies in the hands of close collaborations between engineers and clinicians together.

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Chapter 66

Periodontal tissue engineering and regeneration

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Introduction

Periodontitis is an infectious disease caused by pathogenic microorganisms in the subgingival biofilm, in combination with other risk factors, such as aging and cigarette smoking. Untreated periodontitis results in irreversible destruction of the tooth-supporting tissues, including periodontal ligament (PDL), alveolar bone, and cementum, and may lead to early tooth loss [1]. It has also been linked to many systemic disorders, such as diabetes, rheumatoid arthritis, neurodegenerative disorders, and cardiovascular complications, and has been associated with adverse pregnancy outcomes [2,3]. Consequently, periodontal disease significantly impacts a patient's quality of life and financially impacts both patients and the public healthcare system. Blocking the progression of periodontitis has been achieved by mechanically removing bacterial biofilm with scaling and root planning procedures. Although the elimination of periodontal pathogens may improve clinical attachment levels (CAL) of a tooth, reconstruction of the support and function of the affected tooth attachment apparatus represents an important therapeutic endpoint that has been an ongoing challenge in clinical periodontitis treatment [4]. Conventional treatment is generally unable to promote the regeneration of the damaged periodontal structures. Although numerous periodontal regenerative treatments have been established, such as techniques that use bone grafting, guided tissue/bone regeneration (GTR/GBR), enamel matrix derivative (EMD), and platelet-rich plasma (PRP), a positive outcome is difficult to predict, and in principle, these existing therapeutic paradigms fall short of reaching a consistent and complete regeneration of the periodontium, particularly in cases where the disease has caused large defects in the periodontal tissue [5].

The recognition that periodontal tissues possess the capacity, albeit in a very limited manner, to repair/regenerate has led to substantial efforts focused on understanding the biological basis for this activity and employing the accumulated knowledge to devise tissue-engineering technology that predictably promotes functional periodontal regeneration, that is, the restoration of all components of the periodontium and the reestablishment of their appropriate connections [6]. Tissue engineering, which is a term often used interchangeably with regenerative medicine, merges the fields of life sciences and engineering biotechnologies and aims to orchestrate body regeneration by specifically controlling the biological environment or developing biological substitutes to restore, maintain, or improve tissue function. The "engineering" of irreversibly damaged tissues is progressing toward reality by improvements in our understanding of wound repair and recent advancements in mysenchymal stem-cell (MSC) biology and biomaterials science. Such advancements help to target molecules and pathways in an effort to restore the regenerative capacity of tissue, and the periodontium could be considered a prime candidate for such endeavors [7]. Applied to periodontal therapy, tissue engineering offers the opportunity to improve periodontal regeneration in a more predictable and qualitative but possibly less invasive manner than currently available regenerative procedures [4,6].

It is an inherent belief in the current concept of periodontal tissue engineering that naturally occurring tissue regeneration can be reproduced in vitro. Clearly, a tissue-engineering approach to periodontal regeneration will need to utilize the regenerative capacity of stem cells residing within the periodontium (or other related tissues) and would involve the isolation of such cells and their

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subsequent proliferation/differentiation within a three-dimensional (3D) framework followed by implantation into the defect [7]. The in vitro design of such therapeutics primarily considers the delivery of appropriate cells in sufficient quantity, the sustained release of chemical and mechanical signals that are often popularly termed growth factors (GFs) in a near-physiological level, and the engineering of extracellular matrix (ECM)-mimicking biomaterials with such desirable properties as macrostructures and microstructures, biodegradability, and biocompatibility [6]. The scaffold not only acts as a delivery vehicle for the cells to the site of regeneration, but it also plays an essential role in cell attachment, space retention, determination of morphological features, and recruitment of oxygen and nutrients [8]. After in vivo transplantation, blood supply, mechanical loading, and pathogen control will become the critical factors that influence the predictability of final outcome. New vascular networks promoted by angiogenic signals provide the nutritional base for periodontal tissue growth and homeostasis, while appropriate mechanical loading would be essential for the development of highly organized, functional PDL fibers [9]. Finally, because of the microbial load at the periodontal lesion, strategies to control infection and host response are required to optimize periodontal regeneration [4].

We must be aware that periodontal tissue engineering is now still in its early stage, with no current paradigm that takes all the aforementioned factors into proper consideration. If periodontal regeneration relies on the in vitro conditioning of a cell-seeded construct for implantation, the transplants should be designed to incorporate biochemical and biophysical signals that recreate important in vivo stimuli, albeit in simplified form, to create a tissue functionally equivalent to native periodontal tissue in terms of composition, biomechanical properties, and physiological performance [10,11]. Although in vitro periodontal tissue engineering has greatly increased our understanding of cellular behavior and cell-material interactions, this methodology is often unable to recreate tissue with the hierarchical organization and vascularization found within the native periodontium [12]. Thus most, if not all, efforts in this field are focusing on in vivo tissue-engineering strategies, in which the traditional triad (cells, regulatory signals, and scaffolds), or a combination thereof, is directly implanted at the damaged tissue site, or within ectopic sites capable of supporting neotissue formation, instead of attempting to recreate tissue replacements/constructs ex vivo [7]. In the broader scope of tissue engineering, cell-free methods can also be considered; nonetheless, it is widely accepted that most of the developing and established strategies rely on the use of cells of different origin. In vivo tissue engineering via cell transplantation or biomaterials design may offer a preferential route for regeneration of periodontal tissue with distinct advantages over in vitro methods that are based on the specific location of endogenous cultivation, recruitment of autologous cells, and patient-specific regenerated tissues [13]. In the future, these exciting developments are likely to help reconcile the clinical and commercial pressures on tissue engineering [12]. However, even if in vivo tissue-engineering strategies are to be used, many issues still that need to be addressed before such strategies become routine in periodontal practice. In particular, the events following cell transplantation are poorly understood. Furthermore, the efficacy and safety of prospective tissue-engineering-based therapies have not been fully evaluated, and the risks have already been underscored by several clinicians and researchers [14].

Approaches to the regeneration of periodontal tissue have made some progress recently, and these paradigms provide useful experimental models for the evaluation of future strategies for treating other connective tissue diseases. This chapter explores the potential cell types, signaling molecules, biomaterials, and various technologies based on the concept of tissue engineering for periodontal regeneration. In addition, possible new directions that need to be exploited to make periodontal tissue engineering a clinical success are highlighted.

Stem cells for periodontal bioengineering

Stem cells are the foundational cells for every organ and tissue, and they have a remarkable self-renewal ability, in that they can divide to replenish any other cell type, thus functioning as a part of a repaired tissue. The healthy PDL harbors stem-cell niches throughout adulthood and exhibits a limited regenerative capacity. However, in a diseased periodontal environment, the lack of robust stem cells renders the use of ex vivo expanded/manipulated stem cells a necessity [6,15]. In this context, periodontal cell therapy involves the treatment of periodontitis by transferring new cells into a defect site with the goal of improving the regeneration process [16]. The transplanted cells may participate in the repair of damaged periodontium, serving as building blocks by differentiating into multiple cell types, or regulate repair via secretion of growth or cellular signals, instead of, or in addition to, directly participating in regeneration of the tissue [17]. Both intraoral and extraoral MSCs have been evaluated for the treatment of periodontal disease and for the regeneration of damaged periodontium [4,14].

Intraoral mysenchymal stem cells

Of note, several cell populations with stem-cell properties have now been harvested from different tissues in and around a tooth and subjected to enrichment and expansion techniques (Fig. 66.1). These populations generally

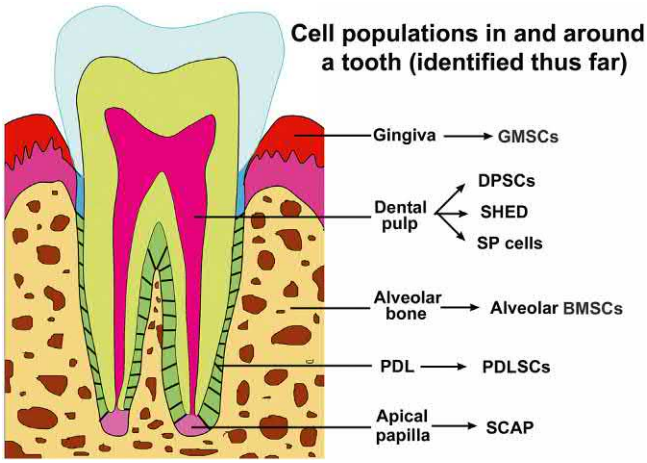

FIGURE 66.1 Selected stem/progenitor cells identified thus far in and around a tooth. PDLSCs, Periodontal ligament stem cells [18]; DPSCs, dental pulp stem cells from pulp of permanent teeth [19]; SP cells, side population cells isolated from dental pulp tissue [20]; SHED, stem cells from human exfoliated deciduous teeth [21]; SCAP, stem/progenitor cells from apical papilla [22,23]; GMSCs, mesenchymal stem cells from gingiva [24–26]; alveolar BMSCs, bone marrow stromal cells isolated from alveolar bone marrow [27,28].

include dental pulp stem cells (DPSCs) [19], stem cells from exfoliated deciduous teeth (SHED) [21], PDL stem cells (PDLSCs) [18], stem cells from apical papilla (SCAP) [22,23], dental follicle stem cells (DFSCs) [29,30], and MSCs from gingival tissues [24,25] and alveolar bone [27,28]. These stem/progenitor cells can be obtained with ease from medical waste, such as teeth extracted for orthodontic, impaction or irreversible periodontitis reasons, exfoliated deciduous teeth, or even gingiva that was removed for hyperplastic or esthetic reasons. The ease of access makes them an important source of autologous stem cells for use in the regeneration of PDL and bone lost in periodontal disease. These stem-cell populations have an advantage over other stem cells, such as bone marrow–derived MSCs (BMMSCs), because they can be obtained from patients in the dental clinic rather than requiring an invasive bone marrow aspiration procedure at a secondary clinic [8]. Because of their particular characteristics of proliferation, differentiation, and plasticity, dental stem cells have also enabled significant progress toward clinical orthopedics and oral maxillofacial bone reconstruction [31–33]. Thus, while not largely exploited, it can be seen that the humble tooth has an important role to play in the development of future regenerative therapies [34].

Periodontal tissue–derived stem cells

When considering application to periodontal regeneration, stem cells derived from the tissues surrounding the teeth, that is, the periodontium should be considered the first choice [14]. The PDL is a fibrous connective tissue that contains specialized cells located between the bone-like cementum and the inner wall of the alveolar bone socket. Just as PDL is essential for osteogenesis and cementogenesis during development and remodeling, cells derived from this tissue are supportive for the healing response to injury [35]. Early observations indicated that the PDL has a regenerative capacity and that a population of multipotent progenitor cells may exist within this tissue [36]. Transplantation of PDL-derived cells has shown the potential to regenerate bone and periodontal attachment apparatus in vivo [37–39]. PDLSCs were first isolated in 2004 and have been shown to give rise to adherent clonogenic clusters resembling fibroblasts [18]. They are positive for the stem-cell markers STRO-1 and CD146, and they are capable of developing into adipocytes, osteoblast-like cells, and cementoblast-like cells in vitro as well as producing cementum-like and PDL-like tissues in vivo [18,40]. In recent years, sources of stem cells from human [41], swine [42,43], and canine PDL tissues [44,45] are reported to be the most potent for periodontal tissue regeneration. Furthermore, in vitro studies have also found that PDLSCs were able to differentiate into mesodermal (i.e., adipocytes, osteoblasts, chondrocytes), ectodermal (i.e., neurons), and endodermal (i.e., hepatocytes) lineages [46]. Meanwhile, PDLSCs have also shown the capacity to differentiate into vascular cells, forming blood vessel-like structures in rats [47]. These findings suggest that the PDL constitutes an important stem-cell source, not only for the regeneration of periodontal tissues, but also for the regeneration of other tissues and organs, making them highly amenable for use in periodontal regeneration. The PDL is under constant strain from the forces of mastication, and thus PDLSCs are likely to play an endogenous role in maintaining PDL cell (PDLC) numbers. This might explain why they are better than other dental stem-cell populations at regenerating the complex structures of the periodontium [40,44,45]. Despite the considerable promise that the PDLSCs hold for periodontal regenerative medicine, outcomes obtained in animals have not been translated into the treatment of periodotitis in humans [48,49]. Hence, a need still exists for further characterization and validation of PDLSCs for clinical use [50].

The alveolar bone is a main part of the tooth-supporting apparatus, functioning as an anchorage of the tooth root to the alveoli and resorbing the forces generated by the function of mastication. Progenitor cells responsible for alveolar bone formation lay in the periosteal region, the PDL, or around the blood vessels. Alveolar bone marrow is considered to be a useful and easily accessible source of bone-related progenitor cells; alveolar MSCs have an osteogenic potential that is similar to, or even more responsive than, cells derived from the iliac crest,

although alveolar MSCs show poor chondrogenic or adipogenic potential [27]. Recently, putative stem cells from human PDL on the alveolar bone surface were isolated, characterized, and provisionally named as "alveolar PDLSCs." Interestingly, these "alveolar PDLSCs" exhibited a strong proliferative capability, expressed high percentages of MSC markers, and were found to have a higher osteogenic/adipogenic differentiation potential than those from the PDL of the root surface [28]. However, further studies need to be carried out to demonstrate that the reported "alveolar PDLSCs" are PDL-derived.

The periosteum is also considered to be a suitable cell source for periodontal regeneration [51]. Mizuno et al. [52] grafted autologous cultured cell membrane that was derived from periosteum peeled from the mandibular body into a mechanically created class III furcation defect in dogs. Proper regeneration of multiple periodontal tissues was observed 3 months after the surgery. In addition, a treatment for human periodontal disease using periosteum-derived cells has already been conducted. It was shown that cultured periosteum combined with PRP and porous hydroxyapatite (HA) granules induced significantly more favorable clinical improvement in infrabony periodontal defects, when compared with the control group (PRP with HA) [53].

The cementum is a specialized calcified substance with ultrastructural similarity to bone that covers the root surface. It is excreted by cementoblast cells within the root of the tooth and is thickest at the root apex. These cementoblasts develop from undifferentiated mesenchymal cells in the connective tissue of the dental follicle at the interface between the dentinal tissue and the PDL, and they contribute to periodontal repair/regeneration after damage. The organic ECM of cementum contains proteins that selectively enhance the attachment and proliferation of cell populations residing within the PDL space [54]. In 1998, human cementum−derived cells were isolated, cloned, and characterized in vitro and in vivo. These cells are capable of differentiating and forming mineralized tissue that exhibits several features identical to cementum when transplanted into immunodeficient mice, although different from the mineralized matrix produced by human BMMSCs [55]. In a proof-of-concept investigation, cementoblasts showed a marked ability to induce mineralization in periodontal wounds in a rodent periodontal fenestration model when delivered to the defects via biodegradable polymer sponges. However, the implanted dental follicle cells seemed to inhibit periodontal healing [56]. These results confirm the selective behaviors of different cell types in vivo and support the role of cementoblasts as a tool to better understand periodontal regeneration and cementogenesis.

The human gingiva is the oral mucosal tissue that surrounds the teeth and forms a mucoperiosteum covering the alveolar bone. Since the epithelial layer shows a unique fetal-like scarless healing process after wounding and has the capacity for continuous renewal, it is anticipated that this tissue could also be a source of stem cells. Recently, cells with MSC properties have been isolated from gingival tissues (GMSCs) and characterized [25,26,57]. GMSCs are easy to isolate and homogeneous, and they proliferate more quickly than BMMSCs in the absence of GFs. GMSCs display a stable morphology and do not lose MSC characteristics at higher passages. In addition, they maintain normal karyotype and telomerase activity in long-term cultures and are not tumorigenic, suggesting that they are superior to BMMSCs for cell therapy in regenerative medicine [24]. Very recently, the in vivo efficacy of utilizing GMSCs in bone regeneration was demonstrated in a mandibular defect as well as a critical-sized calvarial defect model in rats [58]. Furthermore, these cells gave rise to high-quality iPS cells, which suggests that gingival tissue is also a promising cell source for investigating the basis of cellular reprograming and pluripotency for future clinical applications [59].

Stem cells from apical papilla

The apical papilla tissue is only present during root development before the tooth erupts into the oral cavity [60]. A unique population of dental stem cells known as SCAP is located at the tips of growing tooth roots. These cells form adherent clonogenic clusters, and similar to other MSC populations, they are capable of differentiating into adipocytes and odontoblasts/osteoblasts in vitro [23]. By cotransplanting SCAP cells, to form a root, and PDLSC, to form a PDL, into tooth sockets of mini pigs, dentine and PDL were formed [22]. These findings provide support for the use of combined MSC populations in root and periodontal regeneration. Most human tissues from early in their development are not clinically available for stem-cell isolation; however, because roots develop postnatally, the root apical papilla is accessible in dental clinical practice from extracted wisdom teeth. Thus a very active source of stem cells with embryonic-like properties can be readily obtained. Further experiments on the properties of these cells obtained from human teeth following expansion in culture are needed.

Dental follicle stem cells

The dental follicle is a loose mesenchymal tissue surrounding the developing tooth germ that participates in the formation of periodontal progenitor cells. It is believed that this tissue contains stem cells and lineage-committed progenitor cells or precursor cells for cementoblasts, PDLCs, and osteoblasts [61]. The presence of stem

cells in the dental follicle (DFSCs) of human third molar teeth was demonstrated in 2005 [30], suggesting an alternative population of dental stem cells capable of differentiating into cells of the periodontium [62]. These fibroblast-like, colony-forming, and plastic-adherent cells express stem-cell markers (STRO-1 and nestin) and can be maintained in culture for at least 15 passages. STRO-1-positive dental follicular progenitors have been shown to differentiate into cementoblasts in vitro [62] and to form cementum in vivo [29]. Current evidence has shown that immortalized DFSCs were able to recreate PDL-like tissues that expressed periostin, Scx, and type XII collagen, as well as the fibrillar assembly of type I collagen when transplanted into immunodeficient mice [63], while EMD [64] or dentin noncollagenous proteins extracted from dentin [65] could stimulate DFSCs to differentiate into cementoblast lineages. Similar to SCAP, DFSCs represent cells from a developing tissue and might therefore exhibit a greater plasticity than other dental stem cells. However, further research is needed to explore the properties and potential uses of both SCAP and DFSCs.

Hertwig's epithelial root sheath

Considering the difficulty of regenerating functional cementum in periodontal tissue engineering by common practice, some researchers recently have tried to take inspiration from the process of normal tooth root development [66]. Hertwig's epithelial root sheath (HERS) is a bilayer epithelial sheath located at the cervical loop of the enamel organ, which separates dental follicle from dental papilla. Emerging evidence has shown that HERS cells can differentiate into cementum-forming cells via epithelial−mesenchymal transition and induce odontoblast differentiation of dental papilla via epithelial−mesenchymal interaction [67]. As the epithelial remnants of HERS, epithelial cell rests of Malassez are the only odontogenic epithelial cells in mature PDL tissues. Accumulative studies suggested that Malassez play essential roles in maintaining PDL homeostasis, such as prevent ankyloses and root resorption. More importantly, some of its subpopulations harbor features of stem cells, which show the ability to form calcium in osteoinductive microenvironment [68]. Although the study of HERS and Malassez is still at initial stage, these researches can broaden our knowledge on the interactions between tooth development and regeneration.

Stem cells from dental pulp or exfoliated deciduous teeth

The first human dental stem cells were isolated from dental pulp tissue of extracted third molar teeth (DPSCs) and were characterized relative to BMMSCs [19]. DPSCs were found to be highly proliferative, clonogenic cells capable of differentiating into odontoblast-like cells and forming dentin/pulp-like complex when implanted into immunocompromised mice. Subsequently, human MSCs were isolated from exfoliated deciduous teeth and were observed to induce bone and dentin formation in vivo. Furthermore, compared with DPSCs or BMMSCs, SHED grow and proliferate more rapidly and have a higher number of population doublings [21]. Further data demonstrate that pulp side population cells maintain self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis [20]. Although there is no evidence that supports the use of DPSCs to regenerate PDL, they showed the highest osteogenic potential among BMMSCs, PDLSCs, and periosteal cells, indicating that they may form a useful cell source for bone reconstruction around teeth and dental implants [69,70]. In 2009, the clinical trial of alveolar bone reconstruction using DPSCs was successfully carried out, and the data suggest that a DPSC/collagen sponge biocomplex can completely restore human mandible bone defects [31]. Interestingly, the implantation of autologous SHED has been applied to regenerated whole dental pulp in human teeth with injured pulp necrosis [71]. Taken together, these results suggest a potential role for DPSCs in bone and dentin regeneration; however, significant further work is required in this area.

Extraoral mysenchymal stem cells

Extraoral MSCs, that is, nondental stem cells, such as BMMSCs and adipose-derived stem cells (ASCs), have also been investigated as alternative cell sources for periodontal regeneration and bioengineering [72−75].

Bone marrow−derived mysenchymal stem cells

BMMSCs are the most widely investigated MSCs in tissue engineering and regenerative medicine because they are easily accessible, their isolation is straightforward, they can be biopreserved with minimal loss of potency, and they have shown no adverse reactions to allogenic versus autologous MSCs transplants. These cells are clonogenic and have demonstrated the ability to form bone and cartilage in vivo. Because of their differentiation potential, BMMSCs have been used in various phases of clinical application, particularly in orthopedics [76]. Indeed, BMMSCs can efficiently regenerate not only bone tissue but also periodontal tissue in various animal models [77]. Recent data have shown that bone marrow progenitor cells can communicate with dental tissues and become tissue-specific mesenchymal progenitor cells to maintain tissue homeostasis [78]. Animal experiments have demonstrated that autotransplantation of BMMSCs

induced periodontal regeneration in experimental class III furcation defects, and that the defects were regenerated with cementum, PDL, and alveolar bone in the MSC-atelocollagen groups. Less periodontal tissue regeneration was observed in the control group than in the MSC-atelocollagen groups [72]. In addition, transplanted BMMSCs labeled with green fluorescent protein (GFP) were detectable by immunohistochemical analysis 4 weeks after transplantation. The periodontal defects were almost regenerated with periodontal tissue. Cementoblasts, osteoblasts, osteocytes, and fibroblasts of the regenerated periodontal tissue were positive for GFP, which suggested that transplanted BMMSCs could survive and differentiate into periodontal tissue cells, resulting in an enhancement of periodontal tissue regeneration [79]. Moreover, clinical evidence indicated that periodontal tissue could be successfully regenerated using autologous BMMSCs combined with PRP [32]. Significantly, when evaluated in periodontal defects in dogs, cryopreserved MSCs showed no altered regenerative capacity compared with freshly isolated MSCs when applied to periodontal regeneration [80]. Notably, a few clinical cases showed the successful treatment of challenging periodontal defects with a novel cellular allograft that contains native MSCs and osteoprogenitor cells [81]. These data suggest that bone marrow is an excellent candidate source of MSCs for periodontal regeneration. Nonetheless, the established shortcomings in relation to the isolation of BMMSCs, that is, pain and morbidity associated with bone marrow harvest, complicated procedures, as well as low harvested cell number, all mean that the use of this cell population in periodontal regeneration in the clinical setting will be a significant challenge in the future. Consequently, researchers have shifted their efforts on assessing alternative sources of MSCs for periodontal therapies [8].

Adipose-derived stem cells

Adipose tissue is an abundant source of MSCs. ASCs can be readily harvested in large quantities and can be obtained with relative ease with low donor site morbidity. ASCs have adipogenic, myogenic, osteogenic, and chondrogenic potential, and they are very angiogenic in nature. These multipotent cells can be utilized in regenerating diseased or damaged tissue throughout the body, including the periodontium. More importantly, the safety and efficacy of ASCs have been demonstrated in numerous preclinical studies, supporting the use of these cells in curing human disease. Although ASCs originate from mesodermal lineages, recent preclinical studies have demonstrated that the use of ASCs in regenerative medicine is not limited to mesodermal tissue but can also extend to both exodermal and endodermal tissues and organs [74]. Advances in ASCs in regenerative medicine during the past 10 years have paved the way to improved periodontal regeneration. For example, 8 weeks after the transplantation of ASCs mixed with PRP into rat periodontal defects, new PDL-like and alveolar bone−like structures were formed, which implies that ASCs could promote periodontal regeneration in situ [75]. In a canine model of periodontal tissue regeneration, ASCs were transplanted with PRP into dental root bifurcation defects. ASC and PRP transplantation were found to prevent epithelial invasion into the defect area after 4 weeks, while newly induced bone was observed at the site of implantation after 8 weeks [74]. Recently, freshly isolated, uncultured stromal vascular fraction of adipose tissue [82], syngeneic or allogeneic adipose−derived cells [83,84] all have been suggested as a source of autologous progenitor/stem cells, offering a practical, promising candidate for future periodontal tissue engineering or cell-based periodontal therapy.

Selection of cell types

Although both dental and nondental MSCs for periodontal regeneration are promising (Table 66.1), we must be aware that mesenchymal cell populations from different tissues display distinct biological properties [14]. Even if they carry common genetic markers, they are likely to be conditioned by their specific microenvironment and to be committed toward a specific differentiation pathway. Dental stem cells are isolated from specialized tissue with potent capacities to differentiate into odontogenic cells. However, they also have the ability to give rise to other cell lineages similar to, but different in potency from, that of BMMSCs [60]. When comparing different tissue-derived stem-cell sheets for periodontal regeneration in a canine 1-wall defect model, PDLSCs resulted in more newly formed cementum and well-oriented PDL fibers than other cells (BMMSCs and alveolar periosteal cells). In addition, nerve filament was observed in the regenerated PDL tissue only in the PDLC group. The amount of alveolar bone regeneration was highest in the PDLC group, although it did not reach statistical significance among the groups. These results indicate that PDLSCs combined with a β-tricalcium phosphate (β-TCP)/collagen scaffold serve as a promising tool for periodontal regeneration [45]. In another dog model with advanced periodontitis, PDLSCs were also demonstrated to be the most favorable candidate for cell therapy when compared to DPSCs and DFSCs. Autologous PDLSCs showed the best regenerative capacity of PDL, alveolar bone, and cementum as well as peripheral nerve and blood vessel [44]. Except for PDLSCs, it remains to be determined which source of MSCs will be most suitable for regenerative periodontal therapy. The use of dental follicular cells is

TABLE 66.1 Potential cell types for periodontal tissue engineering.

Cell type	Cell association	Therapeutic application	References
PDLSCs[a]	Xenogenous	Rat/ectopic model	Seo et al. [18]
	Autologous	Swine/periodontitis model	Liu et al. [42]
	Allogenous	Swine/periodontitis model	Ding et al. [43]
	Autologous	Dog/1-wall defect model	Tsumanuma et al. [45]
	Autologous	Dog/apical involvement defect	Park et al. [44]
	Autologous	Human/periodontitis	Feng et al. [41]
SCAP[b] & PDLSCs	Xenogenous/ Autologous	Rat/ectopic model; Minipig/in situ (lower incisor extraction socket)	Sonoyama et al. [22]
DFSCs[c]	Allogenous	Rat/ectopic model	Yokoi et al. [63]
	Autologous	Rat/ectopic model	Wu et al. [65]
DPSCs[d]	Autologous	Dog/periodontitis model	Park et al. [44]
BMMSCs[e]	Autologous	Dog/Class III defects	Kawaguchi et al. [72]
	Not defined	Dog/Class III defects	Hasegawa et al. [79]
	Autologous	Dog/periodontal fenestration defects	Li et al. [80]
	Autologous	Dog/Class III defects	Wei et al. [85]
	Allogenous	Rat/periodontal defect	Yang et al. [86]
	Autologous	Dog/1-wall defect model	Tsumanuma et al. [45]
	Autologous	Dog/apical involvement defect	Park et al. [44]
	Allogenous	Patient / periodontal defect	McAllister [81]
ASCs[f]	Autologous	Rat/periodontal palatal defects	Tobita et al. [72]
	Syngeneic	Rat/bacteria-induced periodontitis	Lemaitre et al. [83]
	Allogeneic	Micromini pig/periodontal defects	Venkataiah et al. [84]

[a] *Periodontal ligament stem cells.*
[b] *Stem cells from apical papilla.*
[c] *Stem cells in dental follicle.*
[d] *Dental pulp stem cells.*
[e] *Bone marrow mesenchymal stem cells.*
[f] *Adipose-derived stem cells.*

likely to be limited by the availability of tooth germs, and nondental stem cells may require the transfer of genes to enhance their odontogenic potential. Although the best stem-cell source is yet to be identified, the prospect of using these cells for regeneration represents a step forward in the development of more predictable biologically based therapies for the periodontium [77].

Signaling molecules

GFs mediate crosstalk between cells and their microenvironment via their autocrine and paracrine effects [87]. They initiate their action by binding to specific receptors on the surface of target cells and their chemical identity, concentration, duration, and context contain information that dictate cell fate for safe and effective regeneration of functional tissue. Hence, the importance of GFs in tissue engineering is unsurprising, considering their importance for tissue regeneration [88]. Accordingly, stimulation of osteogenesis, cementogenesis, and connective tissue formation via presentation of various signaling molecules is necessary for periodontal tissue engineering. A number of recombinant human GFs, biological agents (e.g., EMD), and a patient's own biologically active products (e.g., PRP) are now used clinically or are currently under extensive investigation, and some of them have been examined by controlled clinical studies or randomized controlled clinical trials.

Types of signals

Bone morphogenetic proteins

The bone-healing process initiated by a single molecular species of bone morphogenetic proteins (BMPs), members of the transforming GF (TGF)-beta superfamily such as BMP-2 or BMP-7, sets in motion a cascade of cellular events resulting in differentiation of progenitor cells into phenotypes involved in periodontal regeneration [89]. Current data suggest that BMP-2, BMP-4, BMP-7 (i.e., osteogenic protein-1, OP-1) and BMP-12 are potent inducers of bone formation during mandibular reconstruction, with OP-1 (occasionally BMP-2) inducing substantial cementogenesis. In this context, BMP-12 supports the reestablishment of the PDL, including regeneration of cementum and functionally oriented fibers, and prevents ankylosis and root resorption following replantation of teeth (reviewed in [89,90]). There is mounting evidence that recombinant human BMPs stimulate periodontal regeneration if applied with suitable carriers [91,92]. However, the optimal effects are modulated by a range of factors that need careful evaluation in clinical studies. Furthermore, we must be aware that severe side effects, such as ankylosis and root resorption, have also been reported [93], although these were not always in accordance with outcomes obtained by other groups [94]. Considering the results of studies indicating that the application of recombinant human BMP-2 around a periodontal defect induces bone formation but not cementum formation [95], the combined use of BMP-2 and BMP-7 might be suitable for regenerating two hard tissues: bone and cementum [96]. In addition, BMP-2 has also been used in combination with other GFs, such as insulin-like GF (IGF)-1, to synergistically enhance differentiation of PDLCs [86,97] or recombinant human β-nerve GF [98] to improve the quality and quantity of regenerated bone. Of note, the absorbable collagen sponge (ACS) carrier containing recombinant human BMP-2 is now commercially available as InFuse Bone Graft (Medtronic, Minneapolis, MN, United States) and has been applied for sinus floor augmentation and for localized alveolar ridge augmentation following tooth extraction in patients.

Platelet-derived growth factors

Platelet-derived GF (PDGF), as a serum GF for fibroblasts, has five isoforms (AA, AB, BB, CC, and DD); of these, PDGF-BB was found to be more potent than the other isoforms in promoting mitogenesis of PDLCs [99]. At concentrations of 10−20 ng/mL, in vitro studies suggested that PDGF-BB stimulated the proliferation of fibroblasts and osteoblasts [100,101], whereas a higher concentration (≥50 ng/mL) is required for the adhesion of PDL fibroblasts to diseased roots; concentrations of 5−20 ng/mL were not effective [102]. PDGF has also been shown to be effective when combined with either IGF, as shown by significant bone fill upon reentry into the defects in animal models [103] and in patients with periodontal disease [104], or with dexamethasone [105], the latter being a well-known osteogenic differentiation factor. PDGF and IGF-1 had additive effects on calvarial DNA synthesis, but PDGF opposed the stimulatory effect of IGF-1 on collagen synthesis, and IGF-1 prevented the PDGF effect on collagen degradation [100]. In further research, IGF-1 alone at a dose of 10 μg did not significantly alter periodontal wound healing, while PDGF-BB alone at the same dose significantly stimulated new attachment, with trends of effect on other parameters. For example, the PDGF-BB/IGF-1 combination resulted in significant increases in new attachment and osseous defect fill above vehicle at both 4 and 12 weeks [106]. In humans, the use of purified rhPDGF-BB mixed with bone allograft resulted in robust periodontal regeneration in both class II furcations [107] and interproximal intrabony defects [108]. Based on those preclinical and clinical studies, recombinant human PDGF-BB homodimer in β-TCP is approved for the treatment of intrabony and furcation defects, as well as gingival recession in periodontal disease and is commercially available as Gem-21 (Osteohealth Co., Shirley, NY, United States). Furthermore, a large multicenter randomized controlled trial study of PDGF-BB homodimer, together with β-TCP, in the surgical treatment of a 4 mm or greater intrabony periodontal defect demonstrated significant increases in CAL reduced gingival recession at 3 months postsurgery and improved bone fill when compared with those of β-TCP alone at 6 months [108]. The safety and effectiveness of this product was further demonstrated recently in a double-blind, prospective, parallel, active-controlled, randomized, multicenter clinical trial involving 54 patients with periodontal osseous defects [109]. Further longitudinal clinical data are still needed to confirm the long-term effect of this product [110]. Although far from ideal for meeting the needs of complex periodontal therapy, the road from basic research to clinical applications of PDGF-BB or BMP-2 suggests a potential use of protein-based therapeutics for stimulating and accelerating periodontal tissue healing and bone regeneration [111].

Fibroblast growth factor-2

Fibroblast GF (FGF)-2 is a heparin-binding protein that regulates cellular functions including migration, proliferation, and modulation of ECM production, in the wound healing cascade [112]. In periodontal regenerative therapies the in vivo effectiveness of FGF-2 has been evaluated in beagle dogs as well as in nonhuman primates

(reviewed in [113]). The results of these studies suggest that FGF-2 induces significant periodontal tissue regeneration with new cementum and new alveolar bone formation. Recently, a double-blind, placebo-controlled clinical trial (Phase II) was conducted in 253 adult patients at 30 Japanese dental facilities. The data obtained demonstrated that FGF-2 was efficacious in regenerating periodontal tissue without clinical safety problems [114]. Further clinical trials (Phase III) are still needed to establish the clinical efficiency and value of FGF-2 products [115].

Growth/differentiation factor-5

Growth/differentiation factor (GDF)-5 is a member of the TGF-β superfamily, which shows a close structural relationship to BMPs and plays critical roles in skeletal, tendon, and ligament morphogenesis [116]. In the periodontal field, it has been reported that cells involved in root- and PDL-forming stages exhibit significantly stronger signals of GDF-5 in comparison to those in more mature, well-established tissues [117]. In vitro studies have shown that recombinant human GDF-5 at concentrations of 10−1000 ng/mL inhibits alkaline phosphatase activity in human PDLCs [118]. Recently, the effect of recombinant human GDF-5 on periodontal repair was demonstrated in animal models by using various biomaterials as vehicles for delivery, such as ACS, β-TCP or an injectable poly-lactide-*co*-glycolide acid (PLGA) composite (reviewed in Ref. [119]). The combined results suggest that recombinant human GDF-5 appears to safely and effectively support periodontal wound healing/regeneration in intrabony periodontal defects without complications, while in a dose-dependent order. Recently, a Phase IIa randomized controlled clinical and histological pilot study evaluated recombinant human GDF-5/β-TCP for periodontal regeneration in 20 chronic periodontitis patients [120].

Platelet-rich plasma

The use of PRP, a platelet concentrate from autologous blood, is one of the strategies available for modulating and enhancing periodontal wound healing and regeneration [5]. PRP includes a pool of GFs such as TGF-β, vascular endothelial GF, PDGF, IGF-1, epidermal GF, and FGFs. For this reason, it has been suggested that the use of PRP might increase the rate of bone deposition and bone quality in such dental treatments as sinus lifts, placement of autogenous mandibular bone grafts, implants, and periodontal surgery. Both preclinical and clinical studies demonstrate the effectiveness of PRP in bone and periodontal augmentation when used in conjunction with bone graft materials (reviewed in Ref. [5]). Unfortunately, the literature on the topic is contradictory and the published data are difficult to sort and interpret. For example, in a series of clinical studies performed by Döri et al. on the healing of intrabony defects, the addition of PRP failed to improve the total outcomes in terms of probing depth reductions and CAL gains [121]. Furthermore, PRP may not provide any additional effect when associated with GBR around dental implants [122]. Although data published thus far suggest that PRP does not always exert additional effects, a systematic review of literature does find evidence for beneficial effects of PRP in the treatment of periodontal defects. Nonetheless, evidence for beneficial effects of PRP in sinus elevation appeared to be weak [123]. While PRP has been somewhat beneficial when used in periodontal or implant regeneration, it has fallen out of favor recently because of the lack of controlled clinical trials providing strong evidence of its efficiency [124]. However, the use of a patient's own biologically active proteins, GFs and biomaterial scaffolds for therapeutic purposes has opened a new way of understanding regenerative medicine. These simple and cost-effective procedures may have a potential impact in reducing the economic costs for standard medical treatments, soon achieving a "golden age" by the development of user-friendly platelet concentrate procedures and the definition of new and efficient concepts and clinical protocols [5].

Enamel matrix derivative

EMD, an extract of porcine immature enamel matrix, is regarded as a candidate protein mixture that is thought to be the induction of proliferation, migration, adhesion, mineralization, and differentiation of cells in periodontal tissue [125]. EMD contains over 95% amelogenin with small amounts of enamelin and other proteins. In 2000, a randomized, double-blind, placebo-controlled, split-mouth study was designed to compare the clinical and radiographical effects of EMD treatment with that of placebo-controlled treatment for intrabony defects. The results demonstrated that treatment with flap surgery and EMD, compared to flap surgery with placebo, produced a significantly more favorable clinical improvement in intrabony periodontal defects [126]. A commercial EMD, marketed as Emdogain (Institut Straumann AG, Basel, Switzerland), received US Food and Drug Administration (FDA) approval and has been available for clinical application for over 10 years, leading to a number of clinical trials performed to evaluate the clinical effects of Emdogain (reviewed in Ref. [5]). In principle, EMD, which seems to be safe, was able to regenerate lost periodontal tissues in previously diseased sites based on clinical parameters and was better than open flap debridement (OFD) alone or GTR. A systematic review indicates that a combination of EMD and PDGF-BB together with β-TCP can improve the treatment efficacy of intrabony

defects [127]. Combined with allograft materials, EMD may be of additional benefit, but it needs to be further investigated [128]. Thus far, however, no evidence indicates that more teeth could be saved, and no evidence points to any important differences between EMD and GTR [129]. Of interest, a 10-year investigation following treatment of intrabony defects with EMD, GTR, EMD plus GTR, or OFD indicated that the three regenerative treatments showed a statistically significant higher CAL gain, compared with that of OFD at both one and 10 years [130]. However, another double-blind randomized clinical evaluation of EMD for the treatment of proximal class II furcation involvements showed that the use of EMD did not promote a superior reduction in probing pocket depths or a gain in clinical and osseous attachment levels, but only resulted in a higher rate of class II to class I furcation conversion [131]. It seems that the clinical application of EMD for periodontal therapy still requires additional well-controlled clinical trials. EMD is applied not only for periodontal surgery but also for tooth transplantation [132] and management of periimplantitis [133]. However, the clinical predictability of such treatments remains to be determined.

Stem cell–derived exosomes

Exosomes are nanosized extracellular vesicles (40–150 nm) secreted by almost all kinds of cells and play key roles in intercellular communication by transferring their cargos containing mRNAs, miRNAs, and proteins to the recipient cells. The contents of exosome cargos are largely determined by the origin and functional status of their parental cells. Therefore stem cell–derived exosomes can bear the therapeutic effects and can be used as the substitutes of stem cells to promote the regeneration of various tissues. Accumulative studies have demonstrated that exosomes are immune-tolerant and can exert various positive effects on the cellular behaviors of stem cells, such as cell migration, proliferation, and differentiation [134]. Moreover, emerging evidence has indicated that exosome can also speed up angiogenesis and prevent the fibrosis to improve wound healing [135]. In addition, the contents of exosomes can be conveniently enriched by packing exosomes with miRNA, genetic material, and other small bioactive ingredients due to their highly efficient delivery of cargos [134]; hence stem cell–derived exosomes have great potentials for tissue repair and regeneration. In periodontal regenerative therapies the in vivo effectiveness and safety of exosomes has been evaluated in ligature-induced rat periodontitis. The results have indicated that exosomes secreted by adipose-derived stem/stromal cells resulted in significantly higher area of newly formed periodontal tissues than adipose-derived stem/stromal cells [136]. Of note, the mechanisms underlying the therapeutic effects of stem cell–derived exosomes have not been illuminated yet. Further clinical trials are also required to confirm the efficiency and safety of clinical utilization of exosomes.

Crucial delivery barriers to progress

The biological basis of using GFs in periodontal tissue engineering stems from their inherent property of inducing chemotaxis and/or mitogenesis of mesenchymal and somatic cell populations, initiating a cascade of events that ultimately lead to proliferation and differentiation of these cells. BMP-2/-7, GDF-5, FGF-2, and PDGF have been intensively investigated in preclinical and clinical trials and the results appear promising [89,90,111,113,119]. However, at this point in time, it appears very difficult, if not impossible, to translate knowledge about the functions of GFs in embryonic development, tissue formation/homeostasis, and bone healing into clinically applicable solutions that address the loss of periodontal tissue. First, GF therapy is currently hampered by the lack of a safe and efficient delivery system that can provide a sustained therapeutic effect without cytotoxicity or unwanted side effects as a result of using very high doses of GFs [137]. Second, enormous costs are associated with recombinant human GFs in relation to the relatively small and non-life-threatening periodontal defects for which other treatment options (e.g., implants) exist. Of note, the delivery strategies commonly used today in periodontology are bolus injection or physical combination of GFs with prefabricated biomaterials. The rapid degradation, hence low local availability of GFs, suggests that these delivery strategies do not meet the physiological requirements of periodontal tissue repair processes [138]. Thus in spite of the many recombinant GFs and cytokines now available for research purposes and the testing of some in humans, the clinical experience has, so far, been disappointing. Consequently, sophisticated approaches must be developed to deliver GFs that allow for the controlled, sustained, and localized delivery of proteins [88]. New drug delivery systems that regulate the biological presentation of GFs represent an attractive new generation of therapeutic agents for the treatment of a wide variety of diseases. Such devices essentially allow the loaded GF(s) to be released at a desired rate and concentration and to remain at injury sites for a sufficient time to recruit progenitors and stimulate the tissue healing processes [138] (Fig. 66.2). In addition, drug delivery systems, termed cell scaffolds, can be formulated to have particular structures that facilitate cellular infiltration and growth. To improve their properties, many drug delivery systems serve dual purposes. In addition to providing cell support, cutting-edge scaffolds biologically interact with adhering and invading cells and effectively guide cellular

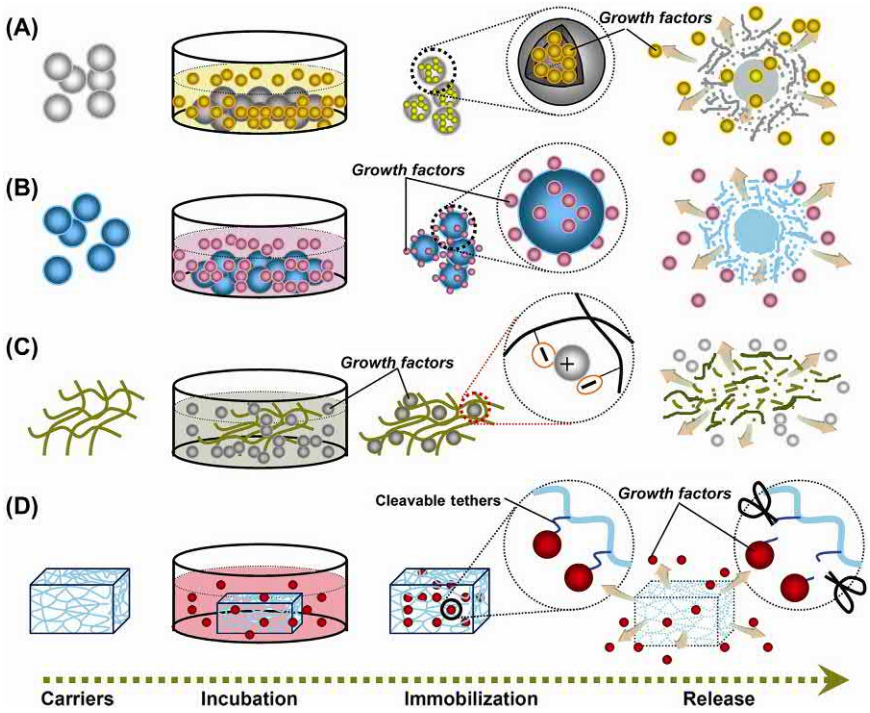

FIGURE 66.2 Schematic illustrations of the frequently investigated methods for immobilization of GFs and their primary release mechanisms (illustration is not to scale). (A–C) Noncovalent immobilization: GFs are directly entrapped into (A) or adsorbed onto (B) the carriers, or immobilized via the formation of ionic complexes with polymer matrices (C). (D) Covalent immobilization: GFs are modified and thereafter covalently crosslinked to the hydrogels via crosslinkable and cleavable bonds that can be liberated once the tethers are degraded hydrolytically or enzymatically. *GFs*, Growth factors. *Reproduced from Chen FM, Wu LA, Zhang M, Zhang R, Sun HH. Homing of endogenous stem/pregenitor cells for in situ tissue regeneration: promises, strategies, and translational perspectives. Biomaterials 2011;32:3189–209 with the permission of Elsevier Inc.*

growth and development by releasing bioactive proteins. To design controlled release systems for specific applications, it is important to understand the basic principles of protein delivery and the stability of each applied biomolecule [140].

Many biological and engineering challenges in the use of GFs for tissue regeneration remain to be solved. It is certain that these signaling molecules promote periodontal tissue and bone formation, but the ideal concentrations still need to be determined, and safety issues currently remain. In addition, understanding when to manipulate the cell's differentiation pathway with the application of single or multiple doses of GFs at the appropriate concentration is required to optimize the effect of these agents in periodontal wound healing. It also seems that any treatment aiming to mimic the natural tissue regeneration processes should not be limited to the provision of a single GF but should deliver multiple agents at an optimized ratio in a specific spatiotemporal pattern [87]. Therefore different release profiles from the same carrier may be particularly important in tissues with mixed cell populations, such as those found in the periodontium, where similar tissues, like bone and cementum, grow at different rates [140]. Furthermore, treatment of intrabony defects with GFs is likely to require both appropriate temporal release of the agents and a carrier that can serve as a template for new tissue formation, providing space maintenance and supporting the mucoperiosteal flap. Many of these issues have not been adequately addressed, at least from a periodontal standpoint; elucidation of these factors is essential prior to conducting expensive human clinical trials.

Gene delivery as an alternative to growth factor delivery

Because of the short half-life of topically administered GFs in vivo, usually ranging from several hours to several days, researchers have tried to extend protein activity using gene delivery strategies that involve converting cells into protein-producing factories. This is achieved by delivering plasmid DNA encoding the GF(s) of interest into cells/tissues either directly or via gene delivery vehicles or vectors [141]. This therapeutic concept has emerged as a promising strategy for the modulation of the host response triggered by periodontal microbe and the regeneration of periodontium during disease progression [4].

Using viral vector transduction, cells can be manipulated in vitro and when transplanted into patients, these cells might restore normal tissue function. One of the most promising gene therapy approaches for periodontal regeneration, however, is based on the combination of naked DNA with a biodegradable carrier. Using this approach, collagen or other biomaterials are used to engineer a so-called gene-activated matrix that can carry and deliver naked DNA that directs an individual's own cells to produce a therapeutic effect [142]. A gene therapy product does not need to carry and deliver manipulated

stem/progenitor cells; rather, it is able to signal the available cells to differentiate into a phenotype more favorable to the regenerative process [143].

Viral vector genes can be expressed in vivo from weeks to years, thereby having greater sustainability than that of a single protein or compound application. A unique advantage of gene therapy is the possibility of delivering more than one regenerative factor by employing two or more DNAs [144]. This combinatorial approach has shown significant regenerative potential compared to individual gene delivery, offering strong potential in regenerating tissues in three dimensions at the tooth−ligament−bone interface. In addition, gene therapy has also been investigated for the possibility of modulating periodontal disease progression via delivery of either antimicrobial or host modulators, considering that the host response against pathogenic bacteria is a major cause of periodontal tissue destruction [145]. Based on this concept, gene delivery, in combination with tissue engineering, is the only possible strategy that may mimic critical aspects of the natural biological processes occurring in periodontal development and repair [4,10,11,146−148]. The genes of interest in periodontal therapy include, but are not limited to, BMPs, PDGF, FGF-2, IGF-1, and TGF-β (reviewed in [141]). Of interest, the adenovirus encoding the PDGF-B gene delivered in a collagen matrix exhibits acceptable safety profiles for possible use in human clinical studies [149]. Although hundreds of gene therapy clinical trials have taken place in the past 20 years, much work still needs to be done to ensure an ideal safety profile for each gene and delivery method combination. With further efforts in this field, it is expected that gene therapy may augment current protein-based strategies and potentially allow for tissue-specific targeting and delivery of multiple signals in a spatially controlled and bioavailable fashion with the goal of regenerating natural tissue replacement in the craniofacial and periodontal region [143].

Scaffolding and biomaterials science

The management of periodontal defects began with the introduction of a "filler" material that aimed to induce bone regeneration. Various types of bone grafts, such as synthetic filler materials (alloplastic materials), autografts, allografts, and xenografts, have been investigated for this purpose. Although utilization of such grafting materials for periodontal defects may result in some gain in CAL and radiographic evidence of bone fill, careful histological assessment usually reveals that these materials have little osteoinductive capacity and generally become encased in dense fibrous connective tissue [7]. Frequently, these treatments serve to ease the symptoms, not cure the disease. Tissue engineering offers clear merits over conventional replacement therapies that suffer from a host of drawbacks, such as scarcity of donor source, donor site morbidity, risk of lateral transmission of pathogens, and graft-versus-host rejection. A major unmet challenge in tissue engineering today has been the synthesis of complex constructs that are principally comprised of biomaterials and multiple cell types. This development is also true in periodontal tissue engineering. With the possibility of therapeutic cell cloning becoming a reality, there is an urgency to develop technologies that can precisely control the behavior of stem cells in culture. Central to these technologies would be the probable inclusion of biomaterials as an important component because the scaffold plays a vital role in converting isolated cells into functional tissues. Advances in biomaterial research will undoubtedly facilitate the transformation of this concept into reality. Factors of great importance in the selection of a suitable scaffold material include such properties as porosity, tissue conductivity, biocompatibility, and resorption rate. An appropriate scaffold has several design criteria that ensure not only the precise delivery of therapeutic cells to the site of injury but also the rigorous control of their fates in vitro and in vivo toward regeneration.

Requirements of cell scaffolds

The implantation of cells in the afflicted area could be a direct approach in regenerative strategies, but the requirement for a support material to promote regeneration, especially in large sized defects, is a critical consideration. Periodontal tissue and bone are expected to grow into an adjacent defect only when the space is maintained and soft tissue ingrowth is prevented. Thus the scaffold should act in a manner consistent with the established principles of GTR. To maintain the space for a sufficient time, the scaffold needs to feature such properties as ease of clinical handling and shaping and sufficient rigidity to withstand soft tissue collapse into the defect [7]. In addition, the scaffold should be biocompatible with no biological hazards, and it should assist tissue functionality by promoting the easy diffusion of nutrients, GFs, and cellular waste products. Furthermore, its biodegradability should be adjustable to the time required for tissue regeneration, thus avoiding interference with the physiological healing process. When considering the compatibility of internal structure with cell attachment and colonization and the compatibility of ingrowth tissues with those to be regenerated, the amount of porosity and the pore size of the supporting 3D structure are important features in the design of tissue-engineering scaffolds [150]. ECM components organized in the PDL not only reflect the functional requirements of this matrix, such as mechanical stress and storage of signaling molecules, but also regulate the tissue framework during development and regeneration.

This idea is inspired in nature itself because most cells in the body subsist in a 3D world and are anchored onto a network of ECM, which the scaffolding design proposes to recreate. With this in mind, scaffold characteristics should mimic the complex and demanding environment to which cells are exposed and should be able to define a local biochemical and mechanical niche with complex and dynamic regulation that cells may sense, while, at the same time, not inducing an overexpression of inflammatory response [151]. Moreover, biosafety and bioactivity are of great importance. Although no guidelines have yet been established for assessing the safety and efficacy of a biological transplant, clearly the materials should be free from transmittable disease and immunologically inert [7].

Biomaterial-based immune modulation

Although a set of newly designed parameters (e.g., geometries, architectures, and mechanical properties) used for finely tuning the cellular behaviors of stem cells has been introduced into the field of biomaterials, limited success is achieved when the in vitro studies were translated into the in vivo experiments [12]. The reason might be lie in that findings based on certain stem-cell types cannot genuinely reflect the in vivo cell milieu, where multiple cell types are involved in the process of periodontal tissue engineering. Besides stem cells, immune cells have received considerable attention as modulators of disease pathogenesis and tissue remodeling following injury [152,153]. Macrophages can exert either positive or negative influences on the outcomes of the implanted biomaterials depend on their polarization states. Classically, the polarization states of Mφs can be divided into two categories, M1 and M2 Mφs: M1 Mφs can exaggerate proinflammatory responses by producing toxic reactive oxygen intermediates and proinflammatory cytokines, whereas M2 Mφs secrete antiinflammatory cytokines to promote tissue remodeling and healing (reviewed in Ref. [154]). Emerging evidence has shown that finely tuning Mφs toward the M2 phenotype could not only prevent bone loss caused by periodontitis but also exploit the power of stem cells to promote periodontal tissue regeneration [153,155]. These finding suggested that the design of biomaterial phytochemical modifications should also utilize the host immune response to ameliorate the outcomes of medical implants and periodontal tissue engineering therapies [156,157].

Classes of biomaterials

For regeneration, biomaterials are intensively utilized, as a scaffold for cell delivery, a template for cell proliferation and differentiation, or as a capsule/filter to maintain the cell's viability and to deliver the synthesized biomolecules. The resorption, mechanical strength, and efficacy of these materials can be manipulated through structural and chemical design parameters. Biomaterials used in periodontal tissue engineering include natural or synthetic polymers, ceramics, and composites. Each material offers a unique chemistry, composition and structure, degradation profile, and possibility for modification.

Naturally derived polymers

Natural biomaterials serve as a cornerstone in the development of matrix-based regenerative therapies that aim to accelerate clinical application due to their excellent biocompatibility, biodegradability, affinity for biomolecules, and wound healing activity [158]. Collagen, hyaluronic acid, alginate, and chitosan scaffolds have been used in periodontal regenerative research for more than two decades. The natural origin of these materials allows the design and engineering of biomaterial systems that function at the molecular level, often minimizing chronic inflammation. They can also be easily modified, both chemically and physically, to form desired structures, possess optimal properties, and perform specific functions for various applications. The use of natural polymers in the form of hydrogels allows for the incorporation of biological agents by promoting crosslinking when the GF is dispersed in the polymer solution. Because natural polymers are often soluble in water, the creation of hydrogels may occur under mild fabrication conditions that are relatively harmless to the bioactivity of the GFs. Normally, these hydrogels are degraded by enzymes and/or acid hydrolysis at a rate depending on the degree of crosslinking or the molecular weight [158]. In addition, natural polymers can also be modified to become 3D scaffolding materials, such as porous sponges, particles, films, and rods, and for the formulation of stimuli-responsive biomaterials.

Purification of the major component of the ECM has been the subject of many potential tissue engineering strategies (reviewed in Ref. [159]). Collagen is regarded as one of the most useful biomaterials owing to its excellent biocompatibility and safety associated with its biological characteristics, such as biodegradability and weak antigenicity. While collagen hydrogel fits well with injectable cell delivery, highly porous collagen lattice sponges with crosslinking provide mechanical stability that has been used to support the in vitro growth of many types of tissues and to deliver multiple GFs. Following the clinical use of collagen carriers delivering BMPs for tibial shaft fractures [160], spine fusions, and long-bone nonunions [161], collagen is currently being evaluated for widespread clinical periodontal regeneration. Particularly, collagen is used clinically as a composite with ceramics [162]. Commercially available collagen composite scaffolds include Formagraft (Nuvasive, San Diego, CA,

United States) and OssiMend (Collagen Matrix, Franklin lakes, NJ, United States). Apart from InFuse Bone Graft, ACS has been used as a carrier and/or scaffold for periodontal regeneration in animal studies [163] and clinical trials [41].

As a deacetyled derivative of chitin, chitosan is structurally very similar to naturally occurring glycosaminoglycans and is biodegradable in mammals. Chitosan has several merits for use as a cell vehicle material, including its ability to be molded into various geometries (e.g., porous structures) and forms (e.g., gels), ease of chemical modification, high affinity for in vivo macromolecules, and minimal foreign body reaction [164]. While chitosan can support cell attachment for cell delivery purposes, it is not strongly supportive of tissue regeneration, as demonstrated by its effect on the width of keratinized gingiva in dogs [165]. Accordingly, chitosan needs to be either modified chemically or conjugated with other molecules or peptides to enhance its biocompatibility for cell attachment and capability for tissue regrowth [7]. For example, the addition of HA beads to chitosan gels produced a novel scaffold in which the pore sizes and interconnectivity were preserved. When loaded with FGF-2, this scaffold may provide a suitable 3D environment supporting cellular structure, proliferation, and mineralization [166]. Chitosan has also been widely used in combination with collagen [146,167], coral [148], HA [147], and β-TCP [168] to develop new scaffolds for periodontal tissue-engineering applications.

Alginate hydrogels bearing cell adhesion ligands have been used as scaffolds for cell encapsulation and transplantation, and they have yielded promising results in experiments aimed at engineering bone tissue capable of growth from small numbers of implanted precursor cells. An alternative to alginate hydrogels as a cell carrier is the incorporation of cells into beads of alginate to prevent immune cells and soluble complexes from killing the transplanted cells; this property negates the need for immunosuppressant use [169]. As a result of the semipermeable nature of the beads, the soluble factors made by the entrapped cells can be released at the implantation site to guide regenerated tissues [7]. However, these developments have not yet been used in periodontal bioengineering. Of interest, calcium alginate film was found to be more effective for GTR and GBR use than the collagen membrane [170].

Synthetic polymers

Numerous synthetic polymers have been widely used for scaffolding applications. Although synthetic matrices in their native forms lack cellular recognition sites, they have well-controlled and reproducible chemical and physical properties [171]. Synthetic polymers have many advantages. For example, they offer the ability to provide controllable and reproducible structural properties, they are biocompatible, and their biodegradation rates can be tailored for the intended application through specific chemical manipulation. Synthetic scaffolds are produced by a variety of fabrication techniques, and these materials can be easily manufactured into preformed sizes and shapes according to clinical requirements. Synthetic matrices are typically processed into the form of solid scaffolds, small particles, or hydrogels, depending on the mechanical and degradation properties for the particular application [171].

Hydrolytically degradable polymers are widely used materials for scaffold manufacture. The US FDA has approved the use of poly(α-hydroxyester)s, such as poly (glycolic acid) (PGA), poly(L-lactic acid) (PLA), and their copolymers, PLGA and polycaprolactone (PCL), for a variety of clinical applications. Solid scaffolds are typically porous matrices fabricated by techniques such as solvent casting, gas foaming, particulate leaching, and electrospinning [171]. Normally, these materials do not elicit a permanent foreign body response as they are gradually degraded into natural metabolites and eventually replaced by natural tissue. The physical properties of these polymers can be readily altered by varying the ratio of lactide/glycolide, the molecular weight, and the crystallinity, or by combining them with other materials [150]. In some cases, synthetic polymers can be used as additions to natural biomaterials. For example, the rapid degradation of fibrin, a biopolymer critical to hemostasis and wound healing, can be decelerated by modification with poly(ethylene glycol) (PEG), thus creating a hybrid material for cell delivery [172].

PLA is one of the most promising biopolymers, because it is a naturally occurring organic acid, and the monomers may be produced from nontoxic renewable feedstocks [173]. Other degradable polymers include PEG [174], polylactide and polyglycolide [175], and polylactide acetyltributyl citrate and polydioxanon [176], and these polymers have also been explored and tested for guiding periodontal tissue regeneration. Amorphous poly (D,L-lactic acid) is one of the most popular materials considered for scaffold production, and it is used in combination with bioactive glasses because it can be combined with such biomolecules as GFs and antibiotics to establish a locally acting drug delivery system [150]. By this modification, the creation of a macroporous structure within the bioceramic materials is possible. For example, when PLGA microparticles are incorporated into calcium phosphates (CaP) cement, a macroporous CaP cement scaffold is formed after PLGA hydrolysis in vivo [177]. Although synthetic scaffolds have excellent mechanical properties and processing ability, their lack of natural biological cues can be a potential weakness. Furthermore, some

polymers suffer from shortcomings, such as eliciting persistent inflammatory reactions and eroding or being incapable of integrating with host tissues [13]. These issues need to be addressed prior to clinical application.

Ceramic-based materials

Ceramic-based materials have been widely used for bone and periodontal regeneration. Commercially available products include CaPs (e.g., HA and TCP), calcium sulfate, and bioactive glass [178]. Clinically used forms of HA and β-TCP include injectable formulations that harden at body temperature, such as β-BSM (Etex, Cambridge, MA, United States) and BoneSource HA Cement (Stryker, Kalamazoo, MI, United States). These materials exhibit a range of degradation rates that span from weeks to years. Their biocompatibility and high protein-binding affinity make them good vehicles for drug delivery [109]. Recently, several porous ceramic scaffolds have also been examined for their utilization as cell delivery materials [7].

Both synthetic and coral-derived porous HA have been shown to support significant clinical improvements in periodontal measures following implantation in intrabony defects [15]. The porosity and degree of sintering of synthetic HA ceramics primarily determine the rate of biodegradation.

The degradation rate of β-TCP, a porous form of CaP, can be controlled by changing the Ca/P ratio. Because of its degradation characteristics and its similar proportions of calcium and phosphate to cancellous bone, β-TCP is regarded as an ideal material for bone substitutes for periodontal repair [119,179]. Owing to the rapid degradation of β-TCP and its associated poor mechanical properties, research has focused on mixed CaPs, such as mixtures of β-TCP and HA or β-TCP and polymers. These hybrid materials appear to be reliable vehicles for cell delivery, with studies showing good tissue formation associated with the implanted cells [7]. The treatment of periodontal intrabony defects with a combination of two ceramics (calcium sulfate in combination with β-TCP) led to a favorable clinical outcome after two years [180]. The alloplastic material Osteon (Genoss, Suwon, Korea) has an HA surface coated with β-TCP (70% HA and 30% β-TCP). The pore size of Osteon is 300−500 μm, and its volumetric porosity is approximately 77%. It has recently been reported that it is suitable for use in sinus graft applications [181]. For periodontal regeneration in periodontitis models, HA/TCP composite is demonstrated to be effective, both as vehicles for cell delivery [42] and as supporting biomaterials for cell sheet implantation [43].

Biomaterial redesign for periodontal application

The selection of the specific biomaterial is a key variable in the design and development of scaffolding systems. It has recently been proposed that a combination of several materials may offer the best opportunity for beneficial clinical outcomes [137]. For example, a porous chitosan/collagen scaffold [167] and a glycidyl methacrylate derivatized dextran/gelatin scaffold prepared through a freeze-drying process were developed specifically for periodontal tissue engineering. Furthermore, natural polymers can be strengthened mechanically by combining them with ceramics, such as HA [147,166], β-TCP [168] or polylactide (blends, copolymers, and interpenetrated networks) [182]. Thus for periodontal regeneration, one promising direction is the formation of hybrid materials that take advantage of both natural and synthetic materials by combining excellent mechanical properties and an intrinsic structure highly compatible with tissue ingrowth. However, in nearly every case, these materials were adopted from other areas of science and technology without substantial redesign [13]. Although a number of biomaterials, such as ASC and β-TCP, have had a long history of clinical use in periodontal therapy, very few interact with their surrounding host environment or promote regeneration of functional periodontal tissue such that a seamless integration with host tissue is created in an intelligent and proactive fashion. Alongside the rapid advances in stem-cell biology, progress in biomaterials design and engineering is now converging to enable a new generation of instructive materials to emerge as candidates for periodontal regenerative medicine.

In terms of shape and geometry, preformed scaffolds are suitable for defects requiring mechanical strength or a predefined shape, such as one-walled bone defects, whereas injectable scaffolds may facilitate clinical use in two- or three-walled intrabony defects. Injectable types of scaffolds are expected to form the shape of any cavity or repair site but must solidify within a clinically acceptable timescale in situ. Of note, such devices will shorten surgical operation time, minimize damaging effects of large gingival retraction and lessen postoperative pain. One critical disadvantage of injectable biomaterials is their poor mechanical strength and porosity; however, pores can be generated in scaffolds with rapidly degrading particles or gaseous bubbles as porogens to promote cell attachment, migration, proliferation, mass transfer, vascularization, and material resorption [183]. These modified hydrogels are promising delivery systems for therapeutic cells.

The functional significance of the ECM has generally been defined in terms of the provision of a structural support for cell adhesion and the establishment of tissue physical integrity. Recent evidence has, however, led to a paradigm shift where the ECM is increasingly recognized as exerting a profound influence on cell behavior. Therefore biomaterials are required to act as the temporary ECM in the regeneration of a new tissue. To this

end, a scaffold is not simply a mechanical support and initial cell anchorage site, but it must be "informative" to the cells. That is, the scaffold should be capable of stimulating specific cellular response at the molecular level [184]. It follows that the design of new cell seeding scaffolds must take into account microenvironment design features that induce the appropriate gene expression in cells forming new tissues [185]. The complexity of this design process is exemplified by the dynamic states of the cells and tissues, which are regulated by the spatial and temporal coordination of multiple cell processes, each of which is regulated by multiple reciprocal interactions between cells and their ECM [185]. Biomaterial modification can take on different levels of complexity to produce increasing levels of physiological "mimicry" and functionality. Their interactions with cells and tissues may be improved or fine-tuned by adequate chemical modifications, such as grafting with cell adhesion peptide Arg-Gly-Asp (RGD) or by surface treatments (e.g., surface plasma modification or surface chemistry modification through grafting with other polymers) [158,186]. The incorporation of RGD peptides in hydrogel matrices has been found to significantly enhance the attachment, spreading, survival, and mineralization of encapsulated dental follicle progenitors, suggesting that RGD additives may promote the use of hydrogels for periodontal mineralized tissue engineering. The incorporation of peptide motifs recognized by cell receptors and the use of recombinant DNA technology have enabled the creation of naturally derived tissue-engineering scaffolds with new levels of biofunctionality [17]. Typical surface modifications include, but are not limited to, changes in hydrophilicity, functionalization with charged groups, the incorporation of insoluble ligands and peptide cell recognition sequences (e.g., RGD), the attachment of larger proteins, supramolecular self-assembly, and the development of materials that bind and release soluble factors [187]. The wettability of the material surface, particularly in synthetic polymers, can be effectively regulated by physical treatments, for example, by irradiation with ions, plasma, or ultraviolet light. The irradiation-activated material surface can be functionalized by various biomolecules and nanoparticles. This characteristic further enhances its attractiveness for cells and its effectiveness in regulating cell functions [188]. Strategies based on physical cues include the reproduction of nanoscale topology, superposition of mechanical cues, and control of degradation. Designing biological recognition into a biomaterial may also obviate the need for therapies based on the delivery of cells or recombinant GFs, which are subject to regulatory constraints [185]. To this end, scientists are creating new materials, including those with improved biocompatibility, stealth properties, responsiveness (smart materials), specificity, and other critical properties.

We must be aware that sites of injury or diseased organs are characterized by heightened immunological surveillance and a high concentration of inflammatory cytokines. As such, these sites often present environments hostile to healthy cells attempting to establish and repopulate. This is extremely important in the management of periodontal disease. Therefore to eliminate the need to promote a harsh immunosuppressive regime, an additional role for scaffolds needs take into account the insulation of their cellular cargos from the host immune system to, in turn, promote the survival of transplants [4]. Finally, to engineer functional periodontal tissues, the correct mechanical stimuli will need to be conveyed to the developing tissues within the scaffold, although very few studies have addressed this issue [7]. To date, biomaterials are rapidly being developed to display and deliver stem-cell regulatory signals in a precise and near-physiological fashion within the tissue defect, fostering the regeneration of the missing tissue [87]. The transplant must participate in the regenerative process, considering the specific properties of each tissue interface, which can only be achieved through the design of scaffold materials accommodating the specific characteristics of periodontal hard and soft tissues, and providing stem cells with the necessary cues to satisfy cellular needs of both tissue types. Further synergism of cell biological and biomaterials technologies promises to have a profound impact on stem-cell biology and to provide insights that will advance stem cell−based clinical approaches to tissue regeneration [189].

Periodontal bioengineering strategies

Periodontal tissue-engineering strategies typically involve harvesting of suitable autologous donor tissues (e.g., the PDL) from which cells are isolated and expanded in a good manufacturing practice facility. Expanded cells are then seeded onto a matrix and surgically implanted into the host's periodontal defects. Alternatively, the cell/matrix may be further incubated in a bioreactor, or other such system prior to implantation, in which the cells may be exposed to biological, chemical, or physical stimuli that promote the formation of the appropriate tissue [6]. Considerable preclinical data and some clinical successes support the applicability of these cell-based approaches [44,72,74]. However, the process is very complex and not cost-effective. In addition, the scarcity of the cell source is an issue. Although allogenic cell transplantation has shown success [43], the potential risks associated with transplanting exogenous (foreign) cells remain to be addressed. Even without any therapeutic intervention, living periodontal tissues can have a staggering capacity for regeneration. As such, a biomaterials/protein-based strategy that avoids the use of ex vivo cultured stem cells to facilitate endogenous repair is a novel approach to periodontal tissue engineering.

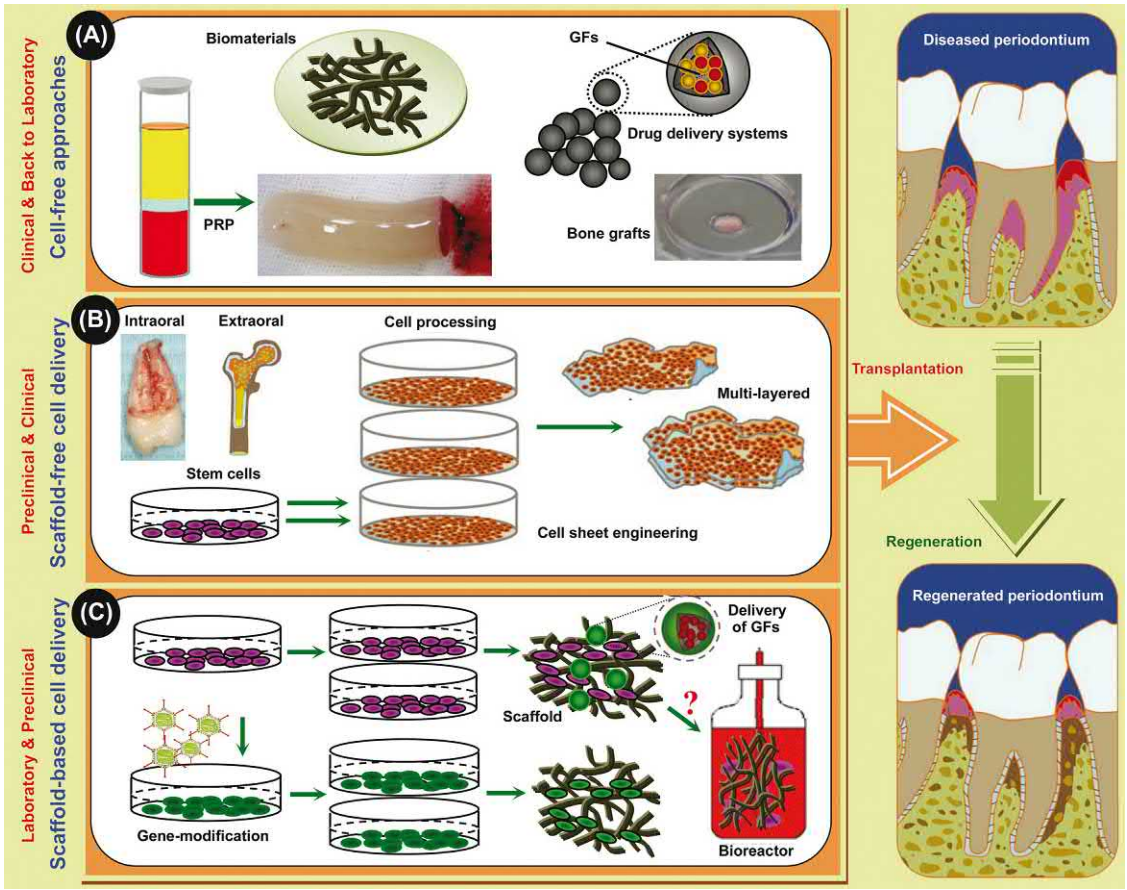

FIGURE 66.3 Cell-free (A) and cell-based (B and C) technologies for periodontal tissue engineering. (A) Cell-free approaches aim at achieving endogenous periodontal regeneration with the use of GFs, well-designed biomaterials, PRP, and its associated formulations, and maybe other commercially available products, alone, or in combination. In this regard, periodontal defects are likely to be restored by endogenously recruited cells and without the need of delivery of ex vivo manipulated cells. This concept has been employed in clinic for several decades and feedback based on clinical practice has led to new insights into and novel applications of such endogenous mechanisms for tissue regeneration [137,139]. Besides, both extraoral and intraoral stem cells represent a viable and accessible "tool" for regeneration. Adequate cell density could be reached in vitro under a controlled environment and made readily available for reimplantation into a periodontal defect site via either scaffold-free (i.e., cell sheet engineering) (B) or scaffold-based delivery (C). Although the time is now ripe to move stem-cell therapy from preclinical studies to clinical trials, there are a number of biological, technical, and translational hurdles that remain unresolved. For scaffold-based delivery the expanded cells are seeded into a 3D scaffold (normally containing GFs), and immediately implant into periodontal defects (in vivo) or to create tissue-like constructs in bioreactor before transplanting into patients (in vitro). In both cases, gene of interest may be incorporated into a therapeutic cell because a therapeutic gene has been shown to increase the regenerative potential and enhance the availability of therapeutic cells. *3D*, Three-dimensional; *GFs*, growth factors; *PRP*, platelet-rich plasma.

Based on current understanding of periodontal tissue engineering, both cell-free and cell-based approaches have their own advantages and disadvantages, and both warrant further investigation (Fig. 66.3).

Cell-free approaches

As noted in the Introduction section, recent advances in periodontal bioengineering have shifted the focus from the attempt to recreate tissue replacements ex vivo (in vitro paradigm) to the development of an in vivo paradigm to achieve in situ periodontal regeneration [6,12]. However, a daunting question for the in vivo paradigm involves the requirement for transplanted cells, as this has been viewed as a core component for in vivo tissue engineering. Interestingly, but not surprisingly, cell-free in vivo tissue engineering has shown that appropriate stimuli can activate local cellular populations and direct their trafficking, leading them to contribute to endogenous tissue formation [5] (Fig. 66.4). The design of cell-free approaches for periodontal regeneration is based on the concept that relies on harnessing the intrinsic regenerative potential of endogenous tissues using a conductive/inductive scaffold, either alone or in combination with such molecular stimuli as GF or genes, to initiate reparative processes in situ [12,139,190,191]

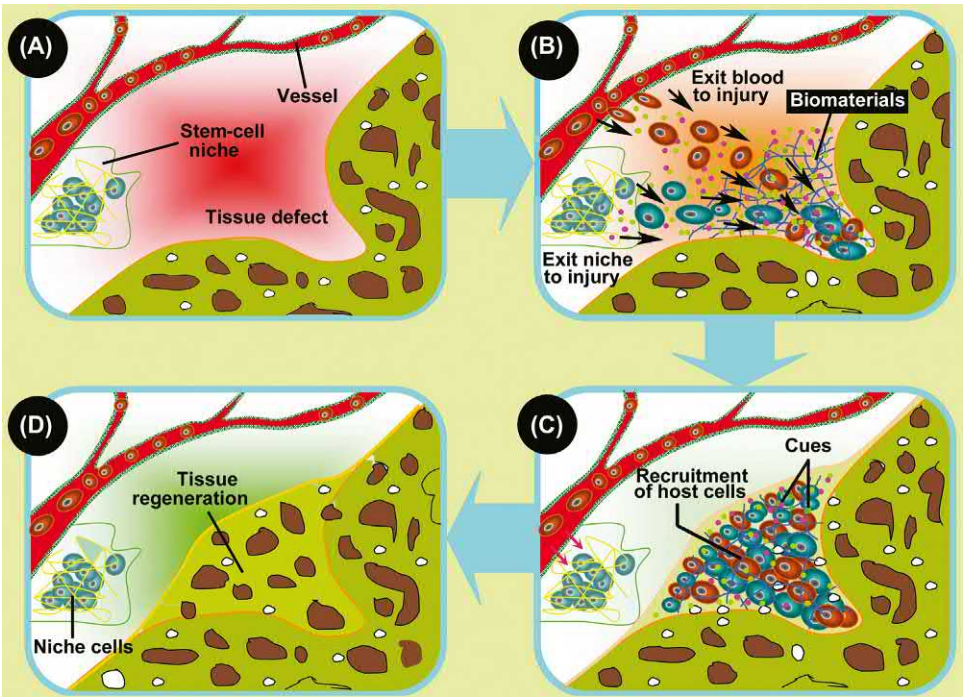

FIGURE 66.4 Schematic representation of the recruitment of host cells for endogenous tissue regeneration. (A) Tissue defect; (B) cell trafficking in response to implantated biomaterials and growth factors; (C) sufficient cell populations recruited to the defect; (D) defect restoration via endogenous tissue regeneration. The use of well-designed biomaterials in combination with molecular stimuli (growth factors or genes) may harness the body's innate ability to restore small or moderate tissue defects without the need of ex vivo cell delivery. This concept is generally termed cell-free tissue-engineering strategy. *Modified from Chen FM, Zhao YM, Jin Y, Shi S. Prospects for translational regenerative medicine. Biotech Adv 2012;30:658–72.*

In carefully selected cases, upon implantation, a conductive scaffold may guide the regeneration of the periodontal tissue by passively allowing the attachment and growth of vascular elements and progenitor/stem cells that reside in the tissue defect. Nevertheless, its regenerative potential is limited by the lack of biologically active factors and sufficient progenitor cells within the defect site. On the other hand, an inductive scaffold may guide the regeneration of the periodontal tissue by carrying one or more biologically active factors, such as stromal cell–derived factor-1, BMP-2, PDGF-BB, and/or FGF-2, which recruit vascular events and progenitor/stem cells from the immediate vicinity to the tissue defect (reviewed in Ref. [13]). The regenerative potential of an inductive scaffold is higher than that of conductive biomaterial, because more progenitor cells can repopulate the tissue defect [192]. Trafficking of stem/progenitor cells requires the complex processes of mobilization of MSCs from their niche, recruitment to the desired target tissue or site, and integration and maturation at the target site. For cell-free tissue engineering to be successful, identification of specific cues that steer stem cells to their niche and increase the efficiency of the homing process is of critical importance [157,193]. For stem cells to be recruited by injured tissues, implanted biomaterials must initiate a sequence of coordinated interactions between the cells and their environment and provide the signals and signposts that guide the cells along their journey [139,156,194]. Therefore artificial biomaterial niches would need to incorporate appropriate navigation cues that could attract endogenous stem cells and have ECM-mimicking components and structures that could localize and accommodate the cells within the destination [6,156].

The use of PRP and EMD in periodontal reconstructive surgery provides evidence that cell-free approaches are useful in various experimental and clinical models, yet the outcomes have frequently been inconsistent or conflicting between studies [6]. If we just consider the current endogenous technique for routine clinical periodontal regeneration, it clearly needs to be improved and refined [195]. One pivotal aspect in the development of PRP technology, for example, has been the deep platelet characterization that has been developed to determine the most important GFs and cytokines contained within these cells, and the protocols that facilitate their safe manipulation and concentration [196]. Cell-free tissue engineering is still in its infancy. New insights from developmental biology and other biological disciplines are actively guiding the development of biofunctonalized materials that work with nature's own mechanisms of repair, where the scaffold should be considered as both a biochemical and biophysical signaling device, modulating and orchestrating cellular activity in the defective microenvironment to harness and direct a patient's own regenerative potential [194]. Although the cell-free approach offers a preferential route for tissue-engineering therapies to be utilized within clinical settings, much remains to be done to illustrate how to administer chemicals that direct stem cells for targeted movement and how to recreate a local

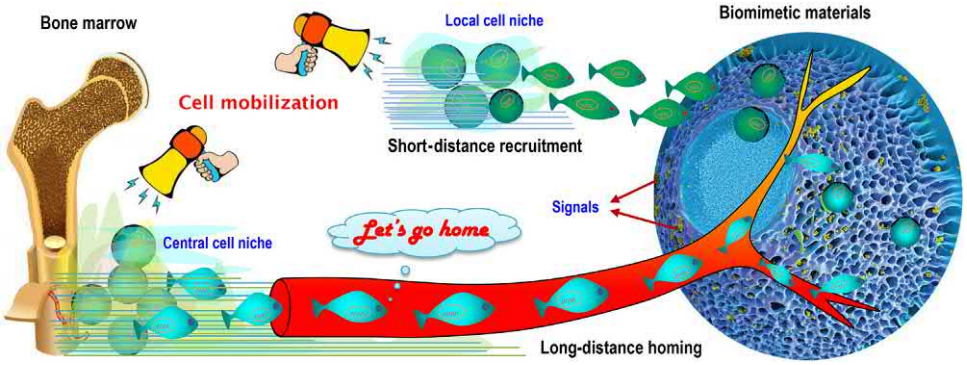

FIGURE 66.5 Schematic representation of how to coax stem-cell homing and recreate a local microenvironment for stem-cell accommodation. The administration of chemicals and growth factors can mobilize resident stem cells from their niche (e.g., the bone marrow) and importantly direct stem cells to move and be recruited to site of injury, wherein biomimetic materials are normally applied to recreate a local microenvironment favoring the recruited cells to live and hence facilitate the cells to regenerate new tissues. *Modified from Wu RX, Xu XY, Wang J, He XT, Sun HH, Chen FM. Biomaterials for endogenous regenerative medicine: coaxing stem cell homing and beyond. Appl Mater Today. 2018;11:144–65.*

microenvironment using biomaterials linked to the immune system to stimulate cell to carry out repairs [156,157,194] (Fig. 66.5).

Cell-based approaches

If an existing degree of periodontal dysfunction already exists and cannot be restored by endogenous mechanisms alone, stem-cell implantation to enhance a regenerative outcome could be pursued as an alternative (Table 66.2). In addition to promoting tissue repair directly, MSCs have also been shown to modulate the immune system and attenuate tissue damage caused by excessive inflammation [6]. This immune modulation has been found to be an active process that renders stem cell therapy.

Scaffold-free cell delivery

Without the use of scaffolds, cells can either be injected directly to the defect or form sheets/microtissues for surgical implantation (reviewed in Ref. [14]). The main drawback of single-cell injection is the high cell loss caused by washout and the low level of integration of the transplanted cells, in addition to the risk of unwanted neoplastic growth if the cells eventually settle in nontarget sites. The development of advanced scaffold-free delivery systems is an emerging subdiscipline of regenerative medicine with broad applications and significant impact in orthopedics and reconstructive surgery. For example, cell sheets/microtissues allow for the generation of functional cell transplants in which cells can survive, grow, and recapitulate native tissue characteristics. In this concept, fibroblasts, or other cell types that secrete significant amounts of ECM proteins, are cultured for a prolonged period to create sheet/tissue-like structures for therapeutic use [115]. Based on the presence of deposited ECM produced during in vitro incubation, the cell transplants can be easily transferred and attached to other surfaces, such as tooth root and host bone tissues. The resulting cell sheets/pellets retain their original ECM and cell–cell contacts to facilitate cell integration and tissue formation.

Because cell sheet engineering can produce functional layers of cells, various types of cell sheet transplantations without scaffolds have been tested for periodontal regeneration, and these have shown significant potential to induce the reformation of the PDL and cementum [39,43,45]. Thus the application of cell sheets may be an effective clinical strategy, and the safety and efficacy were evaluated, both in vitro and in vivo, and presented no evidence of malignant transformation [50,197]. Very impressively, this concept has also been tested in a feasibility/pilot study in which autologous PDLCs were utilized in the treatment of human periodontitis [41]. Recently, a randomized clinical trial in which autologous PDLSC sheets in combination with Bio-Oss was used to treat periodontal intrabony defects has already been performed [48,49]. Although the use of PDLSC sheets in combination with bone graft materials did not produce satisfying therapeutic outcomes, this preliminary study confirmed the safety and feasibility of translation of stem cell sheets utilized in clinical practice. Considering the complex microenvironment of periodontal defects (e.g., oral microorganism, inflammatory, and mechanical stimuli) and the complex structure of periodontium (alveolar–PDL–cementum) to be regenerated, future clinical trials can focus more on ingenious designing of cell sheets, for example, multiple-layered cells sheets with inflammatory or hypoxic pretreatments.

Cell pellets or microtissues are groups of cells (cell reaggregates) normally ranging in size between 100 and 500 μm in diameter. These cell aggregates gain structure and functionality resembling the native tissue when dispersed cells are grown under certain culture conditions [200]. Keeping this in mind, ex vivo expanded dental

TABLE 66.2 Delivery strategies in cell-based periodontal tissue engineering.

Scaffold-free delivery

Design of delivery	Therapeutic cells	Applications	References
Cell sheet engineering	Human PDL[a] cells	Dog—dehiscence defects on the buccal surface of the mesial roots of bilateral mandibular first molars	Akizuki et al. [39]
	Allogeneic PDLSCs[b]	Miniature pig—experimental periodontitis	Ding et al. [45]
	Autologous PDLPs[c]	Human—16 teeth in patients with periodontitis	Feng et al. [41]
		Human—20 teeth patients periodontitis	Chen et al. [48,49]
	Autologous PDL cells, iliac BMMSCs,[d] or APCs[e]	Canine/one-wall intrabony defects	Tsumanuma et al. [45]
	Human PDLSCs	Rat/ectopic model	Iwata et al. [50] Washio et al. [197]
Cell pellets	Autologous PDLSCs, DPSCs[f], or PAFSCs[g]	Canine/apical involvement defect	Park et al. [44]

Scaffold-based delivery

Design of delivery	Therapeutic cells	Biomaterials/applications	References
Injectable carrier or soft scaffold	Autologous PDL fibroblast-like cells	Autologous blood coagulum/dog—artificial fenestration defects	Doğan et al. [38]
	Autologous cementoblasts	PLGA[h] polymer sponges/Rat—rodent periodontal fenestration model	Zhao et al. [56]
	Autologous BMMSCs	Atelocollagen (2% type I collagen)/Dog—class III furcation defects	Kawaguchi et al. [72]
	Autologous cryopreserved BMMSCs	Collagen scaffold carrier/Dog—periodontal fenestration defects	Li et al. [80]
	Autologous ASCs[i]	PRP[j]/Rat—periodontal defects in the first upper molar palatal side	Tobita et al. [74]
	Autologous CDCs[k] and PDL-derived cells	Collagen sponges/Dog—periodontal intrabony defects	Nuñez et al. [198]
Hard scaffold	Autologous PDLSCs	HA-TCP[l]/Miniature pig—experimental periodontitis lesions	Liu et al. [42]
Cell encapsulation	Autologous BMMSCs	Microcarrier gelatin beads/Rat—surgically created periodontal defects	Yang et al. [199]

[a] *Periodontal ligament.*
[b] *Periodontal ligament stem cells.*
[c] *Periodontal ligament progenitors.*
[d] *Bone marrow mesenchymal stem cells.*
[e] *Alveolar periosteal cells.*
[f] *Dental pulp stem cells.*
[g] *Periapical follicular stem cells.*
[h] *Poly-lactide-co-glycolide acid.*
[i] *Absorbable collagen sponge.*
[j] *Platelet-rich plasma.*
[k] *Cementum-derived cells.*
[l] *Hydroxyapatite-tricalcium phosphate.*

stem cells were grafted to the periodontal defect without the need of a scaffold, where the cellular pellets could cover the denuded root surface thoroughly and fill the defect [44]. Furthermore, bilayered cell pellet constructs comprising calcified bone-forming cell pellets (i.e., BMMSCs) and cementum/PDL-forming cell pellets (i.e., PDLSCs) can be fabricated in vitro via the intrinsic capacity of monodispersed cells to self-assemble into a

microtissue (e.g., 3D spheroid). This strategy may provide an alternative to reconstruct extensive periodontal defects and achieve a predictable and complete regeneration of the periodontium. However, the possibility of microtissues turning into tumors based on their structural similarity is a great concern, especially when undifferentiated cell types are used [201].

Scaffold-based cell delivery

From an engineering standpoint, current approaches to periodontal regeneration via scaffold-based cell transplants can be summarized into four possible strategies [14]:

- Stem cells are amplified by ex vivo expansion and differentiated into the target cell type before being seeded into scaffolds to constitute the transplants;
- Stem cells are amplified and differentiated directly in the scaffold before implantation, which is a strategy more suited to adult stem cells, but instructive signals need to be incorporated into the scaffolds to effect direct cell proliferation/differentiation in situ within the scaffolds;
- Stem cells are partially differentiated into progenitor cells, either before or after seeding into scaffolds, to give rise to proto-tissues; following implantation, these constructs transiently release progenitors that migrate into surrounding regions where they undergo terminal differentiation, integrate and contribute to regeneration of the lesion areas (prolonged release of stem/progenitor cells may be achieved when a suitable scaffold is used to maintain them in a partially differentiated state).
- Injectable transplants composed of pristine or stimulated stem cells encapsulated in biodegradable hydrogels; this strategy is attractive for soft tissue repair or treatment of solid tissues with critical size defects that are too fragile for surgical intervention.

All four strategies have been tested in periodontal tissue engineering. Injectable materials are considered to be the ideal delivery vehicles for cells and bioactive factors, because they can be delivered by a minimally invasive process and can fill irregular spaces of the defect. The injectable system can offer early mechanical stabilization by in situ polymerization, such as fibrin and alginate [85]. The rapid degradation of fibrin can be decelerated by modification with PEG, thus creating a hybrid material for robust cell delivery [172]. It is known that PRP may enhance new bone formation and accelerate existing wound healing process. Therefore autologous injectable scaffold was used to deliver BMMSCs to the periodontium [32]. Although cell transplantation via injectable matrices into sites of articular defects has been successful, it has not been tested in either preclinical or clinical studies to demonstrate whether injections of stem cells into periodontal defects can achieve significant improvement.

In contrast, hydrogel sponges as vehicles for cell and drug delivery for periodontal regeneration have been tested in various animal models and in clinical practice with success [158,198]. In terms of synthetic biomaterials, a pilot study has demonstrated that cementoblasts have a marked ability to induce mineralization in periodontal wounds when delivered via PLGA polymer sponges [56]. Impressively, a collagen sponge scaffold has been shown to be an effective cell delivery vehicle for oromaxillofacial bone tissue repair in patients [31].

Solid scaffold-cell transplants may stimulate the repair of damaged/diseased tissue while maintaining adequate integrity, where HA/TCP can be used directly as a cell delivery scaffold [42,43]. The requirements and design of scaffold systems for cell delivery have been discussed previously in this chapter. Many promising strategies for functional tissue engineering aim to replicate components of the natural cellular microenvironment by providing a synthetic ECM and by delivery of GFs [137]. A major clinical challenge in the reconstruction of large oral and craniofacial defects is the neogenesis of osseous and ligamentous interfacial structures where the natural 3D shape of the tissue needs to be recreated. In this regard the multiscale computational design and fabrication of composite hybrid polymeric scaffolds (PCL-PGA) to carry genetically modified human cells to regenerate human tooth dentin−ligament−bone complexes have been tested in vivo. This approach offers potential for the clinical implementation of customized periodontal scaffolds that may enable regeneration of multitissue interfaces required for oral, dental, and craniofacial engineering applications [10,11]. Recently, cell-based research has focused on the geometric design of scaffolds for tissue engineering. However, the orchestration of multiple tissue formation, spatial fibrous tissue organization, and endpoint functional restoration using a single in vivo scaffold system remains a significant challenge.

Stem cells can potentially be expanded on microcarrier beads in spin culture for direct transplantation into tissue defects. The process of encapsulation physically isolates cells from the outside environment and aims to maintain cellular physiology within a selectively permeable membrane [199,202]. By mechanically blocking the cells from immune attack, this technology significantly reduces posttransplantation cell apoptosis and offers a solution to the shortage of donors for functional cell transplantation, particularly since it allows xenobiotic or allogenic cells to be used. However, this therapy has not yet been introduced to periodontics, and it still faces many challenges, such as materials choice and design, before it can be moved into clinical reality [203].

In prospect, shape memory materials are potentially useful for periodontal regeneration, although they remain to be tested. These materials can deliver bulky scaffolds via minimally invasive surgery to the periodontal defects. Using these smart materials, future scaffolds may be fabricated in a condensed state before transplantation and then later acquire the desired shape in vivo [200]. Self-shaped hydrogels can be structurally collapsed into smaller, temporary shapes that permit their minimally invasive delivery in vivo. Scaffolds are rehydrated in situ with a suspension of cells or cell-free medium and delivered through the same catheter. The rapid recovery of the scaffold properties facilitates efficient cell seeding in vivo and permits neotissue formation in the desired geometries [203]. Improved delivery platforms coupled with enhanced understanding of the mechanistic/biological regulators of tissue formation should lead to better and more predictable therapeutics within the next decades [148].

Challenges and future directions

The currently available treatments, which are based on the "damage to heal approaches," have had only limited success in periodontal medicine. With an increasing aging population, tissue-engineering strategies provide important cures and hope for the treatment of periodontal disease, and they have set the stage for successful regeneration of many other tissues. The development of biological transplants for reconstructive therapies has considerably improved the currently available treatment options for periodontal repair. Particularly, the accelerated pace of research in the stem-cell field and the accumulated body of knowledge has spurred interest in the potential clinical use of stem cells [17]. This developing area is attracting increasing attention from both the private and government sectors because of its considerable economic and therapeutic potential. However, there are critical steps in moving the field toward human clinical utility [204]. In particular, the events following cell transplantation are poorly understood, underscoring the considerable need for robust preclinical modeling for the evaluation of the safety and efficacy of stem cells. Although the clinical application of stem cells to the regeneration of periodontal tissue has begun, the risks of stem-cell therapies should not be ignored or underestimated by clinicians and researchers [14].

There are two main criteria for successful tissue engineering [7]. First, there are the engineering principles, which relate to biomechanical properties of the scaffold, architectural geometry, and space maintenance. In this regard, biomaterials are increasingly being developed as in vitro microenvironments mimicking in vivo stem-cell niches. Future successful periodontal bioengineering may very well rest on the ability to effectively utilize these biomaterials as delivery platforms for appropriate cells and/or factors [148]. However, current macroscale methodologies to produce these niche models fail to capture the spatial and temporal characteristics of the complex native stem-cell regulatory systems. The second criterion relates to the biological functions of the engineered construct, including cell recruitment, proliferation, and survival in culture, and at the site of implantation, neovascularization and delivery of GFs necessary for successful differentiation and tissue regeneration. However, control over stem-cell fates within a complex in vivo milieu is extremely difficult and represents the most challenging issue faced by current periodontal bioengineering.

Tissue engineering is making an important impact on the concept of periodontal therapy, and several clinical trials involving transplantation of stem cells into human patients have already begun or are in preparation (reviewed in Refs. [14,204]). The use of protein- or cell-based therapy to enhance and direct periodontal wound healing into a more predictable regenerative path is being exploited in bioengineering efforts that aim at developing a new therapeutic paradigm for clinical application. As tissue engineering becomes more of a clinical reality through the ongoing bench-to-bedside transition, research in this field must focus on addressing relevant clinical situations [204]. Acknowledging that tissue regeneration alone is not the only answer to predictably securing a long-term stable treatment for the patient with a history of periodontal disease, host modulation therapies are therefore rising as an important aspect in the control of periodontal diseases and tissue reengineering [4]. Although most in vivo work in the area of periodontal tissue engineering focuses on tissue regeneration within sterile, surgically created defects, there is a growing need for the investigation of engineering approaches within contaminated or infectious wound beds, such as those that may be encountered following tissue damage by periodontitis. As discussed within the context of this chapter, there are a number of developing systems that have the potential to optimize tissue healing biology. A major obstacle that remains today is how to maximize the utility of therapeutics delivered to a passive or permissive environment where there is a context for the type of cell needed but in which very few biological signals are given to encourage normal cell function [4]. In addition, the tissue-engineering field still needs to confront other hurdles, such as identifying the best cell sources, clinically relevant cell numbers, an effective mode of administration, the integration of new cells into existing tissue matrices, and the achievement of functional properties of tissue equivalents using an expanded repertoire of biomaterials. Finally, major constraints to the tissue-engineering fields remain in the context of practical, safety, and regulatory concerns related to the application of these technologies in the clinical arena [4].

Closing remarks

Regenerative therapies for periodontal disease that use patients' cells to repair the periodontal defect have been proposed in a number of preclinical and clinical studies. Periodontal tissue–derived stem cells, such as PDLSCs, are committed toward all of the periodontal developmental lineages that contribute to cell turnover in the steady-state and would thus be useful cell sources for treating periodontally destructive diseases, such as periodontitis. Treatments that partially regenerate damaged periodontal tissue through the localized administration of GFs have now been established. Although the clinical practice is not very successful, such regenerative therapies have provided very useful and feasible clinical study models for the future design of tissue engineering and stem-cell therapies. Using currently available clinical strategies, partial regeneration of the periodontal tissue is becoming possible; however, methods to achieve the functional regeneration of large defects caused by severe periodontal disease are still lacking. To address this, it is essential to better understand the cellular and molecular mechanisms underlying periodontal development and, thereby, identify the appropriate functional molecules that induce the differentiation of stem cells into periodontal lineage cells for the successful reconstruction of periodontal tissue. The field of periodontal bioengineering has entered an exciting new developmental phase that will make increasingly important contributions to the patient. Particularly, a number of biological technologies are being aggressively explored for clinical translation, signifying a veritable "coming of age" of the field. However, such issues as appropriate delivery devices, immunogenicity, autologous cells versus allogenic cells, identifying tissues that provide the most appropriate donor source, control of the whole process, and cost-effectiveness are all important considerations that should not be overlooked. The future of periodontal bioengineering is undoubtedly driven by technology. New applications and improvement upon current designs will depend heavily on innovations in biomaterials engineering. Progress in stem-cell biology will be imperative in dictating advances in stem cell–based regeneration. A better understanding of the molecular mechanisms by which substrate interactions impact stem-cell self-renewal and differentiation is of paramount importance for targeted design of biomaterials. Discoveries in the fields of developmental biology and functional genomics should also be exploited for broadening the repertoire of biological molecules that can be incorporated into biomaterials for fine-tuning stem-cell activities. With the merger between the two powerful disciplines—biomaterials engineering and stem-cell biology—a new drawing board now lies before us to develop therapies that promise to revolutionize periodontal tissue engineering.

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Part Eighteen

Respiratory system

Chapter 67

Cell- and tissue-based therapies for lung disease

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Introduction: challenges facing cell and tissue-based therapy for the treatment of lung disease

The lung is a remarkably complex organ that evolved for the adaptation of vertebrates to terrestrial life. While embryonic development proceeds normally in the absence of lungs, life after birth requires respiration, a process that is dependent upon the structure and function of the lung. The respiratory tract consists of distinct anatomic regions from the nasal passages, pharynx, larynx, trachea, bronchi, lobar bronchi, bronchioles, and peripheral airways that direct inhaled gases to the alveoli. The distinct architecture of these regions is populated by a great diversity of cell types with distinct functions, (Fig. 67.1) [1].

Lung function is entirely dependent on its remarkable structure that exchanges millions of liters of environmental gases throughout our lifetime. Unlike other organs, for example, endocrine organs that synthesize and secrete hormones critical for the growth and metabolism of many target organs, respiration is an intrinsic property defined by lung structure and mechanics. Acute and chronic pulmonary disorders causing respiratory failure are major causes of morbidity and mortality. There are limited curative therapies for respiratory failure caused by pulmonary disorders, for example, congenital malformations, diffuse interstitial lung diseases (ILD), emphysema, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, and pulmonary hypertension, other than lung transplantation with its accompanying clinical challenges. Regeneration or replacement of functional respiratory tissues remains a long-term goal for ventilatory failure whether related to airway or alveolar disorders. A number of reviews summarize progress in tissue engineering and regenerative medicine relevant to pulmonary diseases [2–4]. New therapies will depend on protecting lung structures that bring environmental gases to an extensive alveolar surface across which oxygen and carbon dioxide are exchanged with the pulmonary vascular bed. Since the pathogenesis in many pulmonary disorders is complicated by defects in mucociliary clearance, and innate, acquired immunity, cell-based therapies will need to account for the highly inflammatory tissue environments characteristic of acute and chronic pulmonary diseases. Achieving tissue or cell-based treatments must overcome formidable technical challenges for regenerative medicine, especially considering the extensive tissue remodeling and inflammation associated with life-threatening pulmonary disorders. Repair/regeneration of tissues in conducting airways (e.g., the repair or replacement of the larynx, trachea, or bronchial cartilage) or the delivery of therapeutic cells or cellular products to the lung parenchyma is also the target of regenerative medicine. This chapter will consider issues regarding the application of cell and tissue-based therapies for the prevention and treatment of lung disease. The development of new therapeutic strategies, for example, the use of endogenous lung stem cells, mesenchymal stem cells (MSCs) or their products, and induced pluripotent stem (iPS) cell-based treatments using tissue engineering will depend upon knowledge of pulmonary morphogenesis and repair of lung structures (Fig. 67.2).

Understanding these processes will be useful in guiding studies in which the principles of lung cell–developmental biology are applied to enhance the treatment of life-threatening pulmonary disorders.

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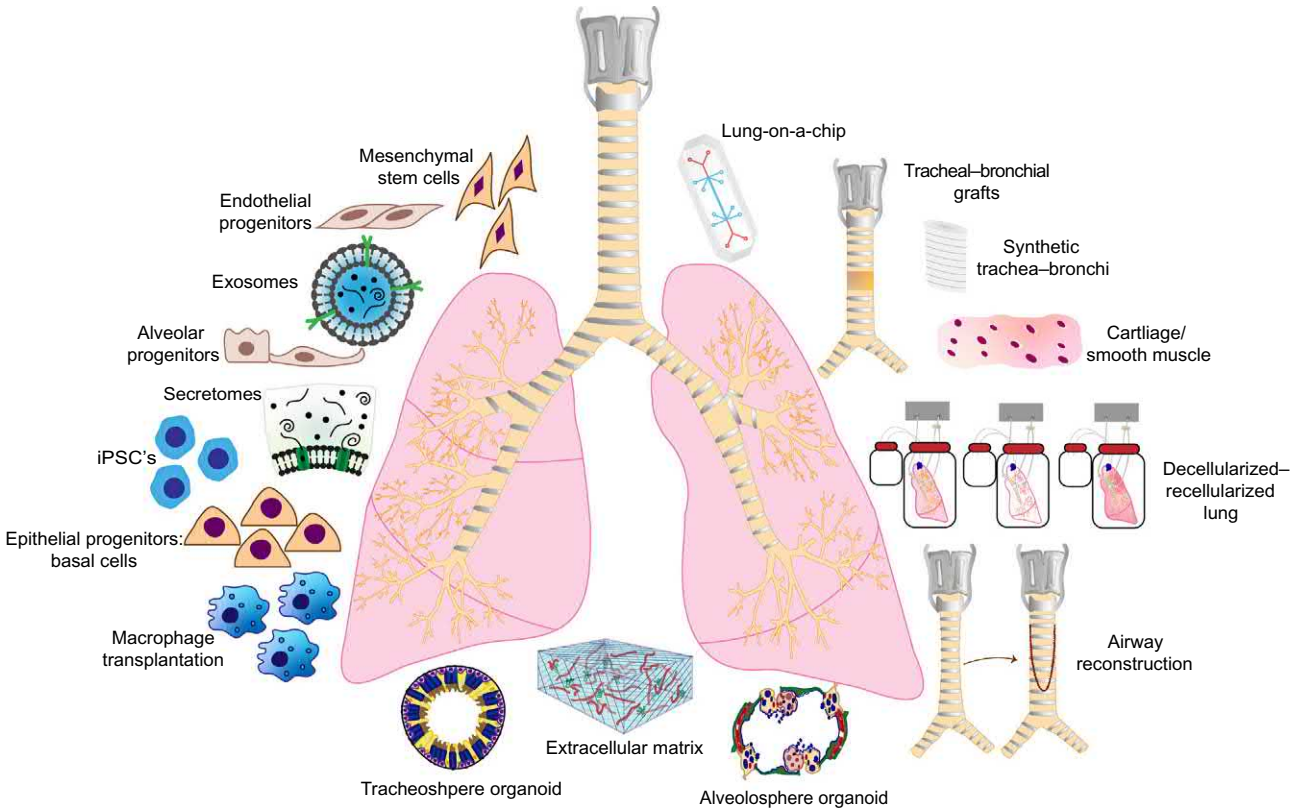

FIGURE 67.1 Strategies for cell and tissue-based therapies for lung diseases. Advances in cell and molecular biology and engineering are being applied to enhance normal lung regeneration or replace cells and tissues for the treatment of life-threatening pulmonary diseases. Research strategies include the delivery of cells, for example, mesenchymal stem cells, lung endothelial, and epithelial progenitors, induced pluripotent stem cells, macrophages or their cellular products, via the airways or the circulatory system. Lung progenitors are seeded on biomatrices, decellularized, and recellularized lung tissues and are being studied at air–liquid interface in perfused and ventilated devices. Tissue engineering and cell-based therapies are being used to produce tracheal–bronchial grafts for the prevention of airway scarring after surgery.

Lung morphogenesis informs the process of regeneration

The lung is a highly complex organ formed by the intricate interplay of dozens of cell types, with contributions from each of the three germ layers. Lung morphogenesis begins as evagination of a small cluster of endoderm-derived cells within the anterior foregut (Fig. 67.3). Epithelial cells of the lung primordia proliferate and migrate into the splanchnic mesenchyme. During, and perhaps even prior to this initial outgrowth of the nascent lung, a complex series of signals are exchanged between the epithelium and mesenchyme which are essential for the proper patterning, differentiation, and outgrowth of each cellular compartment. Signals from epithelial progenitors instruct vasculogenic and angiogenic processes in endothelial progenitors in the splanchnic mesenchyme. Interactions among mesenchymal, vascular, and smooth muscle cells with the epithelial cells produce the basic branched structure of the embryonic lung during the process of branching morphogenesis. Paracrine and autocrine cell interactions are regulated by numerous signaling and transcriptional pathways that instruct cell proliferation, migration, and differentiation. Extensive progress has been made in recent years in understanding the signaling pathways, transcription factors, and molecular and cellular processes by which the trachea, bronchi, and peripheral airways are formed, and are reviewed elsewhere [5–10]. Knowledge of these signaling pathways and transcription factor networks will prove to be a fundamental tool in advancing our ability to repair and regenerate the injured lung [1,11–14].

During early embryonic development of the respiratory epithelium, pluripotent cells are initially programmed to produce endoderm and are subsequently restricted to anterior foregut endoderm. Next, cells within the anterior foregut endoderm are specified to produce lung epithelial progenitor cells that will in turn generate a diversity of respiratory epithelial cells that line the conducting airways and alveoli of the mature lung (Fig. 67.2). Wnt signaling from the splanchnic mesenchyme induces foregut endodermal cells to produce lung rather than esophageal cell progenitors. Suppression of BMP (bone morphogenic peptide) signaling, retinoic acid signaling, receipt of high

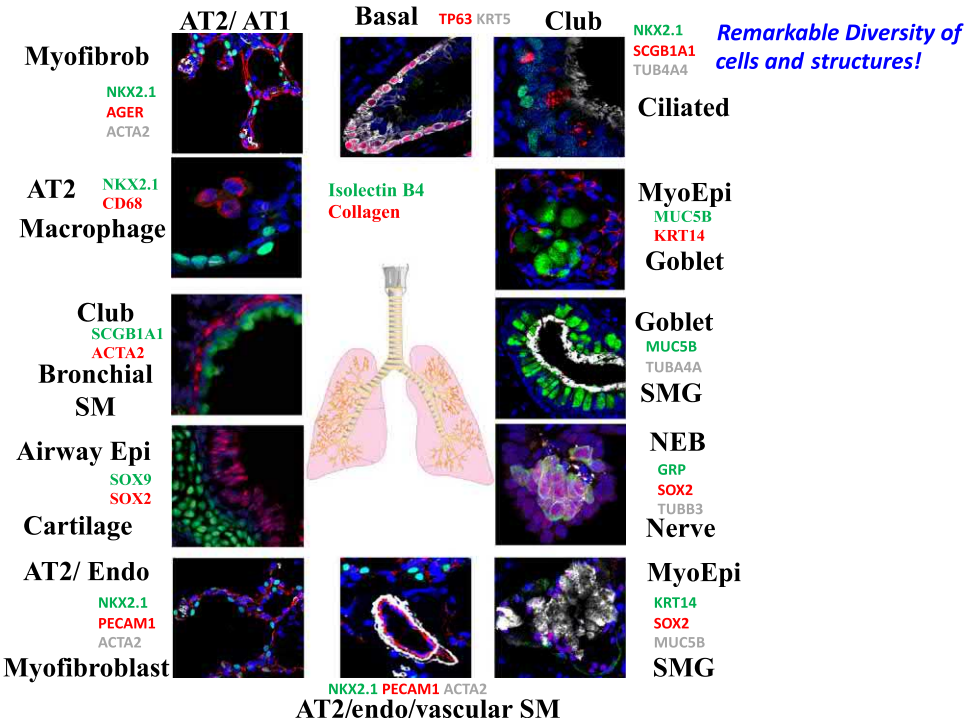

FIGURE 67.2 The complexity of the human lung: a challenge for cell and tissue-based therapy. The respiratory tract from trachea to alveoli is a remarkably complex organ whose structure is critical for respiration. The lung is composed of a diversity of cell types, located in precise anatomical niches that together provide ventilation for the alveolar gas exchange region and the lung periphery. Mucociliary clearance, innate and acquired immunity protect the lung from infection and injury. A diversity of progenitor cells responds to repair the lung after injury and to maintain homeostasis throughout life. Cell and regenerative therapies for lung disorders depend on knowledge of the formation and repair of lung structures, seeking to protect, repair, or replace lung structures critical for ventilation. *Adapted from Whitsett JA, Kalin TV, Xu Y, Kalinichenko VV. Building and regenerating the lung cell by cell. Physiol Rev 2019;99:513–54.*

levels of Fibroblast Growth Factor (FGF), and expression of specific Wnt ligands (Wnt2A/B) serve to specify lung epithelial cell progenitors from those destined to form other organs along the gut tube (e.g., esophagus, thymus, thyroid, the gastrointestinal tract, liver, and pancreas) and initiate branching morphogenesis, [15–24] knowledge useful in directing production of lung cells from embryonic stem (ES) and iPS cells. The commitment, restriction, and differentiation of endodermal cells to form the lung buds are first marked by the expression of NKX2-1 (previously known as thyroid transcription factor-1, TTF-1) [25]. While the trachea and main bronchi are found in NKX2-1 gene deleted mice, branching morphogenesis and differentiation of pulmonary epithelial cell types fail to occur. Complete separation of the trachea and esophagus requires NKX2-1 (thyroid transcription factor 1), SHH (sonic hedgehog), BMP, WNT, and SOX2 signaling, to name a few. In the mouse, lung tubules, stromal, and vascular components typical of the normal lung are lacking in the absence of NKX2-1 [26]. The activity of NKX2-1 is influenced by its interactions with other transcription factors and cofactors that regulate gene expression and differentiation in the various cell types that line the respiratory tract. NKX2-1 is coexpressed with a number of transcription factors, including FOXA2, FOXA1, GATA-6, NF-1, ETV5, SOX2, SOX9, and ETS family members (to name a few), to regulate gene expression and cell differentiation during the formation of the respiratory epithelium [27,28]. Early lung branching morphogenesis in mouse is a highly stereotyped process, and by this point in development, the basic proximal–distal patterning of the lung is established [29,30]. The distal tips of the branching lung contain progenitor cells marked by the expression of SOX9. Early in development, SOX9 + progenitor cells make both airway and alveolar epithelial cells but later during mouse lung morphogenesis, serve as progenitors for the alveolar epithelium [31–33]. In contrast, SOX2 is required for proper differentiation of the conducting airway epithelial cells [34,35]. The precise roles of SOX2 and SOX9, and the mechanisms delineating proximal and distal cell types in the human lung epithelium remain incompletely understood. A recent study identified airway cells coexpressing both of these critical transcription factors, a co-occurrence not observed in mouse [36]. Signaling between the lung mesenchyme and respiratory epithelial cells and between the subsets of cells establishes the number and sites of distinct cell types that line conducting and alveolar regions along the cephalocaudal and ventral–dorsal axes of the lung.

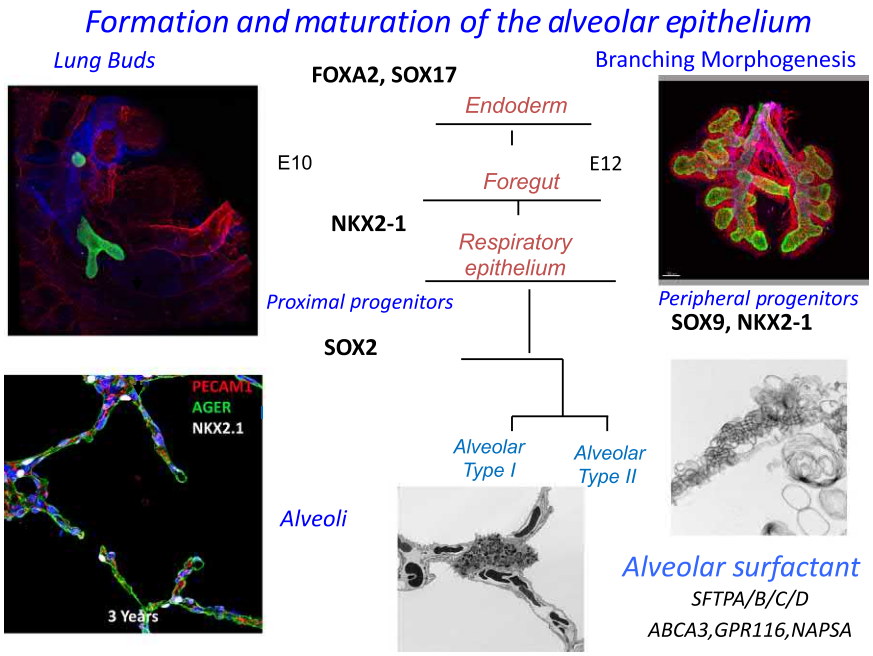

FIGURE 67.3 **From lung bud to alveolar epithelium.** Formation of the embryonic trachea and peripheral lung buds are identified in the fetal mouse embryo on embryonic day 9–9.5 by the expression of NKX2-1 (green) a homeobox gene critical for the formation of the respiratory tract and thyroid (upper left). The lung buds undergo growth and branching morphogenesis and the pulmonary vasculature (red) forms in increasingly close apposition to the epithelial tubules (green) (upper right). Before and after birth the lung undergoes sacculation and alveolarization to create the air spaces and an extensive gas exchange region wherein alveolar epithelial cells (AT1 and AT2 cells) are in close contact with the microvasculature (lower left). AT2 cells synthesize and secrete pulmonary surfactant needed to reduce surface tension and to maintain lung volumes during respiration (shown in the electron micrographs). Sox2, a transcription factor, is critical for the formation and differentiation of the conducting airway epithelium; Sox9 and NKX2-1 program the peripheral lung saccules from which the alveoli are formed during development. *Adapted from Whitsett JA, Kalin TV, Xu Y, Kalinichenko VV. Building and regenerating the lung cell by cell. Physiol Rev 2019;99:513–54.*

Integration and refinement of signaling and transcriptional pathways during lung formation

A number of signaling molecules mediate the autocrine and paracrine signals that are precisely regulated during lung formation. Wnt-β-catenin [15–19], FGF [20,21,37], SHH [38,39], BMP4 [17,22,40], Notch [41–44], retinoic acid [23,45], and Hippo-Yap [46–49] pathways play critical roles during the formation of the lung. Knowledge of the role that these signaling pathways play in the formation of the lung has been a key aspect of recent successes in differentiating human ES cells and iPS cells (iPSCs) into specific lung epithelial subtypes. Moreover, the role that these same signaling pathways play in regulating adult lung progenitor cell populations is an active area of investigation [50,51].

Although many of the core signaling pathways and transcription factors that direct lung development have been defined over the past several decades, mechanisms by which these signals are integrated and retained at the cellular level remain incompletely understood. For example, recent work highlights the role that noncoding RNAs, including microRNAs and long noncoding RNAs, play in providing additional layers of control over gene expression [52–56]. Establishment of proper patterns of chromatin "state," including DNA methylation, chromatin accessibility, histone post-translational modifications, chromatin conformation, and even the large-scale topographical configuration of the genome, are essential for normal development and cellular identity [57,58]. The role that epigenetic modifications play during the course of normal lung development and regeneration of the injured lung is just beginning to be explored.

The mature lung consists of diverse epithelial and mesenchymal cell types

Recent single-cell RNA sequencing identified the remarkable diversity of distinct cell types comprising the lung. For example, the normal mouse lung contains more than 25 major cell types including diverse epithelial, endothelial, mesenchymal, fibroblasts, smooth muscle, and bone marrow–derived cells that comprise the peripheral lung parenchyma [59–64]. The lung epithelium lines the entire airway tree and covers the massive surface area of the alveoli needed to facilitate gas exchange. The cell types present at each point along this proximal–distal

(cephalocaudal) axes of the lung are precisely patterned but vary greatly both in their location and relative distribution between species. Moreover, the number of airway branches, number of submucosal glands, and the extent to which the supporting cartilaginous rings and airway smooth muscle extend down the airway tree vary among species. However, the basic cell types making up the lung epithelium appear to be well-conserved in vertebrates.

The largest and most proximal airways are supported by a thick layer of surrounding mesenchyme, smooth muscle, and cartilaginous rings. The airway epithelium itself is lined primarily by ciliated, secretory, and basal cells but includes relatively rare, but highly specialized epithelial, cell types, including ionocytes, neuroendocrine cells, and brush cells. Neuroendocrine cells can be found as either isolated cells or as organized clusters called neuroepithelial bodies (NEBs), which are primarily located at branch points along conducting airways. NEBs are well innervated and likely serve an important sensory and immune role in the lung and may also provide a niche with unique repair capacity [65,66]. Single-cell RNA sequencing from large numbers of cells have identified brush type cells and ionocytes, which likely play important roles in chemo-sensing fluid and electrolyte homeostasis [67]. Large cartilaginous airways are rich in submucosal glands consisting of multiple epithelial cell types, including myoepithelial cells, goblet, club, and ciliated cells. Submucosal glands secrete fluids, electrolytes, mucins, and host defense proteins critical for mucociliary clearance. Basal cells lining the conducting airway and the submucosal glands are an important progenitor cell population, capable of generating the other airway epithelial cell types in response to injury [68–70]. Smaller, noncartilaginous airways are lined primarily by a more distinct, columnar epithelium consisting of ciliated and secretory cells.

The distal airspaces of the lung are lined by squamous AT1 and cuboidal AT2 epithelial cells. AT2 cells synthesize and secrete pulmonary surfactant required for lung inflation during the respiratory cycle. Lack of pulmonary surfactant in preterm infants causes neonatal respiratory distress syndrome (RDS), a potentially lethal condition. Ongoing dysfunction of AT2 cells after birth can cause diffuse, life-threatening lung disease [71,72]. Alveolar surfaces are lined primarily by AT1 cells, which provide a critical interface between the incoming air and the closely apposed underlying capillary network. Many of the key transcription factor networks coordinating gene expression within these two cell types have been defined with recent new insights gleaned from single-cell RNA-sequencing studies [1].

While cellular diversity within the lung mesenchymal cell populations is less well defined, recent studies have distinguished molecular features of this complex group of cells. Proximal regions of the lung are supported by cartilage, nerves, larger pulmonary arteries, veins, and lymphatics, as well as tracheal–bronchial glands. Pulmonary vessels (arteries, veins, capillaries, and lymphatics) are precisely aligned with the airway lobes and acinar segments and are supported by smooth muscle and a diversity of lung pericytes, fibroblasts, or "stromal" cells. In alveolar regions, mesenchymal cells are less abundant, and endothelial cells within the microvascular network come in close contact with AT1 epithelial cells. The alveoli are also supported by an elastic network consisting primarily of elastin and collagen.

The importance of mesenchymal cells, long thought to be a fairly homogenous population of "supporting" cells, is starting to become better appreciated. Single-cell RNA sequencing and lineage tracing identified Wnt responsive, Pdgfra + mesenchymal cells in alveolar regions, termed mesenchymal alveolar niche cells. These cells are critical for alveolar epithelial cell growth and renewal. In contrast an Axin2 + myofibrogenic progenitor cell forms myofibroblasts and airway smooth muscle cells and contributes to fibrotic responses after injury [73]. LGR5 and LGR6 demarcate distinct populations of mesenchymal cells, with LGR6 marking airway smooth muscle cells supporting bronchiolar epithelial differentiation. LGR5 marks mesenchymal cells supporting alveolar epithelial growth and differentiation [74]. These studies highlight the importance of autocrine–paracrine and direct cell–cell interactions among the various epithelial and mesenchymal cell populations to control proliferation and differentiation during both lung morphogenesis and repair of the adult lung after injury. Mitotic activity of the lung parenchyma is generally high during embryonic and early postnatal development of the lung. In contrast, proliferative rates in the mature lung are remarkably low, cell turnover of lung parenchymal cells occurs over many months in the absence of lung injury.

After birth the diversity of cells with the lungs are strongly influenced by acute and chronic injury. For example, goblet cell and squamous metaplasia are associated with asthma, COPD and other pulmonary disorders. A number of chronic lung diseases are associated with an abnormal expansion of mesenchymal cell populations, for example, in pulmonary fibrosis. Many of pulmonary disorders are also accompanied by inflammation. Dynamic changes in inflammatory cells and their activated following acute and chronic injury play important roles in repair processes.

Structure and function of pulmonary vasculature

Lung is one of the most vascularized organs in the body containing elaborate networks of blood and lymphatic vessels that support respiratory function, Fig. 67.4. Successful

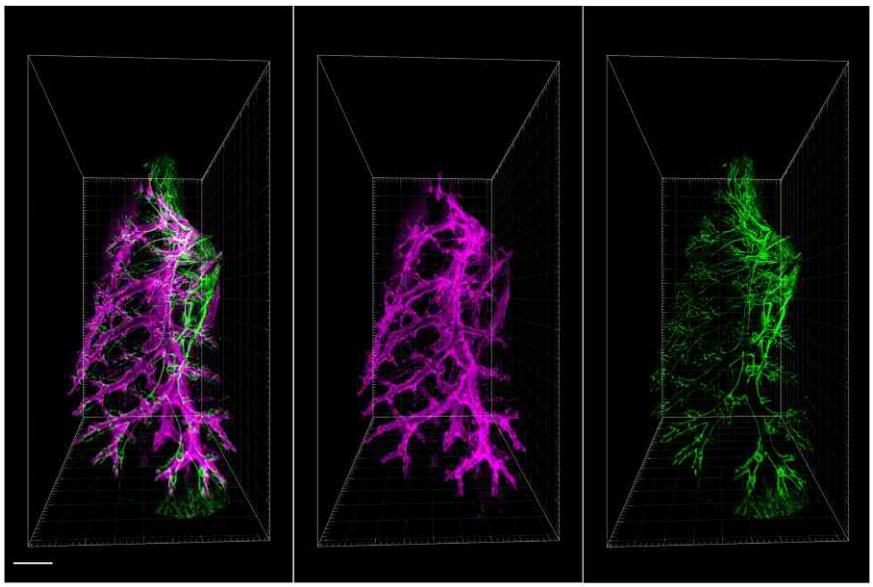

FIGURE 67.4 Complexity of lung architecture shown by confocal microscopy. Secondary harmonic image (purple) identifies collagen in vascular structures of a "cleared" normal mouse lung of 28 days of age (middle panel). Epifluorescence was used to image the airways from trachea to peripheral bronchiolar structure (green, right panel). The overlay of the two images is shown in the left panel demonstrating the bronchial structures and vasculature.

transplantation, cell and tissue-based therapies for the lung, requires the integrity of the pulmonary circulation. The human lung is perfused by both pulmonary arterial vessels, and a distinct bronchial circulation is a part of systemic blood circulation, which is supplied by circulation from the aorta. The bronchial circulation plays a major role in providing oxygen and nutrients to conducting airways. The pulmonary circulation is distinct from the systemic circulation, consisting of pulmonary arteries, veins, and the capillary microvascular networks that create the complex vascular bed within alveolar regions to facilitate gas exchange, Fig. 67.4. Pulmonary arteries deliver deoxygenated blood from the right ventricle of the heart, whereas oxygenated blood from alveoli returns to the left atrium via the pulmonary veins. The lymphatic circulation consists of the networks of lymphatic vessels and blunt-ended lymphatic capillaries that regulate pulmonary homeostasis by draining the interstitial fluid and immune cells into the thoracic duct, which drains into the systemic circulation.

Endothelial cells share the expression of cell surface adhesion molecules CD31 (*Pecam-1*) and VE-cadherin (*Cdh5*), produce PDGFb ligand, and form monolayers covering luminal surface of blood and lymphatic vessels. Arterial endothelial cells express Ephrin B2 (*Efnb2*) and Neuropilin 1 (*Nrp1*) as well as NOTCH 1 and 4, Fig. 67.5 and [75–77]. Venous endothelial cells selectively express Ephrin receptor B4 (Ephb4) and Neuropilin 2 (*Nrp2*), whereas lymphatic endothelial cells express Podoplanin (*Pdpn*), *Lyve1*, and the transcription factor *Prox1*, Fig. 67.5. Pulmonary capillaries consist of a single layer of microvascular endothelial cells that express CD34, FOXF1 transcription factor, and common endothelial markers and form an efficient gas-exchange unit with alveolar type I (AT1) epithelial cells.

Embryonic development of alveolar capillaries

Pulmonary vasculature is formed within mesenchyme located in close opposition to developing respiratory epithelial tubules. Development of vascular networks is dependent on vasculogenesis (de novo formation of blood vessels from endothelial progenitor cells) and angiogenesis (branching of preexisting blood vessels) [78–80], Fig. 67.5.

Large pulmonary blood vessels are generally considered to be formed by angiogenesis. However, recent studies demonstrated that vasculogenesis also contributes to the formation of the main pulmonary arteries and veins during early stages of lung and heart morphogenesis [81]. A common population of cardiopulmonary progenitor cells was shown to give a rise to mesodermal cell lineages that form endothelium, smooth muscle, and pericyte-like cells located in the cardiac inflow tract and large pulmonary vessels [82]. Formation of the microvascular network occurs through the differentiation of endothelial progenitors that initially migrate from splanchnic mesoderm into the lung and later expand within distal lung mesenchyme. Anastomoses between pulmonary blood vessels and the microvasculature occur between E11.5 and E13.5 of lung morphogenesis in the mouse [78–80,83,84]. This process is mainly dependent on leakage-proof remodeling of vessel walls since blood flow without vascular leak was detected as early as E11.5 [83,84]. Circulating endothelial progenitor cells also contribute to the formation of pulmonary blood vessels [85,86].

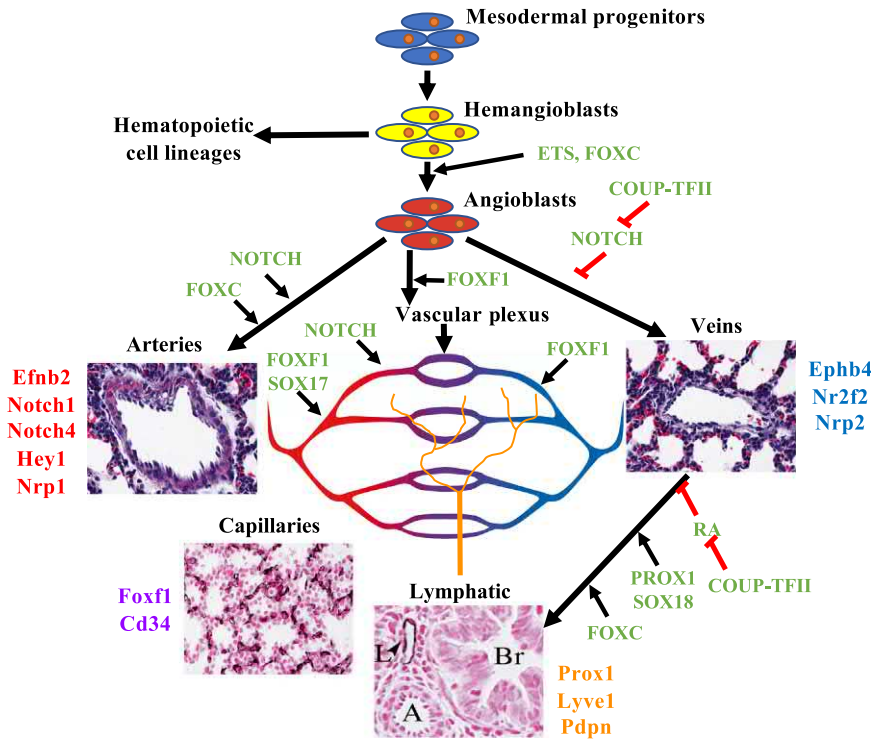

FIGURE 67.5 Embryonic development of arterial, venous, lymphatic, and capillary cell lineages. Schematic diagram shows the diversity of pulmonary endothelial cells. Mesoderm-derived bipotential hemangioblasts give a rise to hematopoietic progenitors and angioblasts (endothelial progenitors). Angioblasts differentiate into arterial, venal, lymphatic, and capillary cells. Differentiation toward arterial endothelial cell fate is dependent on NOTCH signaling. COUP-TFII stimulates venous endothelial differentiation by inhibiting NOTCH. Lymphatic endothelial differentiation is dependent upon PROX1, COUP-TFII, FOXC1/2, and SOX18. Development of pulmonary capillary endothelial cells is induced by FOXF1, SOX17, and NOTCH. Markers of each endothelial cell lineage are indicated next to microscope images. *Adapted from Whitsett JA, Kalin TV, Xu Y, Kalinichenko VV. Building and regenerating the lung cell by cell. Physiol Rev 2019;99:513–54.*

During transition between the saccular and alveolar stages of lung development, the capillary endothelium expands in close apposition to alveolar epithelial cells as AT1 and AT2 cells differentiate. The gas exchange interface is formed between capillary endothelial and AT1 epithelial cells, whereas AT2 secrete surfactant to decrease alveolar surface tension during respiratory cycles. Subsets of AT2 cells serve as progenitor cells during alveolar repair. Lung epithelium produces various growth factors and signaling molecules that regulate growth and expansion of embryonic lung microvasculature. These include VEGF, FGFs, TGF-β, SHH, WNTs. and NOTCH that act through transcription factors, including FOX, SOX, HOX, GATA, KLF, ETS, bHLH, TBX, MEF2, Nuclear receptor, and Zinc finger family members to regulate endothelial proliferation, migration, and survival (reviewed in Refs. [1,81,87–89]). Forkhead Box F1 (FOXF1) is a key transcriptional regulator of alveolar microvasculature [1,81,90]. Loss-of-function mutations in the *FOXF1* gene cause alveolar capillary dysplasia with misalignment of pulmonary veins [91,92], a severe congenital disorder associated with primary loss of alveolar capillaries, malposition of pulmonary veins, and respiratory insufficiency in the first month of life [93]. FOXF1 stimulates endothelial proliferation during embryogenesis through downstream target genes many of which are critical for VEGF, PDGF, NOTCH, and Angiopoietin/TIE2 signaling pathways [81,94,95].

Paracrine signaling between endothelial cells and pericytes is required for the formation of the pulmonary microvascular network. Endothelial cells produce PDGFb and ephrinB2 which signal through PDGF receptor β (PDGFRβ) and Eph receptor B3 (EphB3) that are expressed in pericytes [96]. Loss of either PDGFb or its receptor causes hemorrhaging due to diminished coverage of blood vessels by pericytes [97–99]. Inhibition of ephrinB2 or its receptor EphB3 decreases the number of pulmonary blood vessels and impaired alveologenesis [96,100], whereas intranasal administration of exogenous ephrinB2 prevents the loss of pulmonary capillaries and improved alveolar septation after neonatal hyperoxic injury. PDGFb provides chemoattractant stimulus to PDGFRβ-expressing pericytes as they migrate along newly formed endothelial sprouts to stabilize blood vessels [101,102]. Knowledge regarding the precise temporal and spatial coordination between signaling and transcriptional networks, which facilitate the interactions among pulmonary cell types to form and regulate pulmonary vasculogenesis and homeostasis, will be critical in developing regenerative therapies for pulmonary diseases, including idiopathic pulmonary hypertension.

Evidence supporting lung regeneration

Since the respiratory tract is continuously exposed to pathogens, including viruses, bacteria, fungus, toxic particles, and

toxicants, a remarkable system of innate defense has evolved to maintain pulmonary homeostasis throughout life. This is accomplished by the maintenance of the intrinsic integrity of the epithelial lining, functional mucociliary clearance, production of innate host defense molecules, and the instruction of acquired immune defenses that serve to maintain lung sterility after birth. Since many viruses and pathogens have developed strategies for targeting specific cells, vertebrates have also developed strategies to maintain cells capable of regeneration.

In general, the mature lung is remarkably quiescent. Proliferative rates of all types of pulmonary cells are remarkably slow in contrast to the rapid and ongoing turnover of cells lining the gastrointestinal tract or skin, wherein stem cells proliferate, differentiate, migrate, and senesce within days. Mouse lineage tracing studies suggest that approximately 1% of lung epithelial cells turn over every month of life, suggesting that some pulmonary cells may last the entire life of the animal.

After injury or resection, however, the lung is capable of remarkable proliferative responses, and repair of the respiratory epithelium occurs rapidly to maintain alveolar-capillary permeability and pulmonary homeostasis. Failure to repair is associated with the loss of alveolar epithelial integrity and capillary leak syndromes that can cause acute respiratory failure, as seen in Acute Respiratory Distress Syndrome (ARDS). Repair of the respiratory epithelium is dependent upon endogenous cells that line distinct regions of conducting and peripheral airways rather than bone marrow or progenitor cells produced by epithelial-mesenchymal transition. Epithelial cells that survive injury rapidly spread, migrate, and proliferate to restore the epithelium surfaces. One example is the postpneumonectomy response in rodents after ipsilateral lung resection that is followed by rapid proliferation of many cell types in the remaining lung tissue, serving to regenerate lung volume and function within several weeks after resection [103]. Lung regeneration depends on available space (chest volume), likely indicating that tissue stretch is an important component of the growth response as has recently been clearly demonstrated during development [104]. Although evidence of lung regeneration following pneumonectomy in human is less well documented, there is some evidence that the mature human lung is capable of regeneration, with younger patients demonstrating more robust regenerative capacity than older individuals. Regeneration after other injuries, including both viral and chemical, are reviewed later.

A diversity of lung epithelial progenitor/stem cells is active during regeneration

Immunohistochemistry, lineage-tracing analysis, cell sorting, in situ hybridization, and single-cell RNA profiling are being utilized to identify specific lung epithelial cell types and to identify progenitor cells (see Fig. 67.6). Cell survival, proliferation, and capacity for self-renewal and

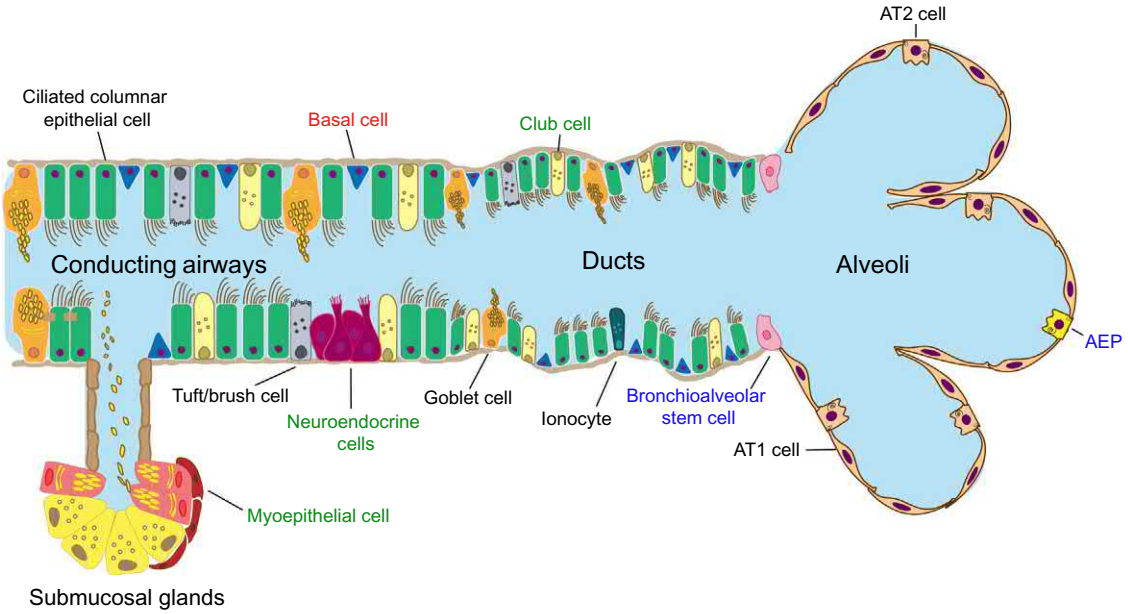

FIGURE 67.6 Progenitor relationships during lung regeneration. A diversity of lung epithelial progenitor cells maintains each area of the lung. The basal cell (*red text*) is a professional progenitor cell for the airway. Club, myoepithelial, and neuroendocrine cells (green) have plasticity and can participate in airway regeneration after certain injuries. The AEP (blue) cell subset of AT2 cells functions as an alveolar progenitor in both homeostasis and after injury, and BASC (blue) are rare cells capable of repairing both airway and alveolus depending on injury location. Cells which are predominantly without repair capacity are noted in black. *AEP*, Alveolar epithelial progenitor; *BASC*, bronchioalveolar stem cells.

differentiation differ amongst various cell types and regions within the lung [105–107]. In conducting airways the epithelium is repaired primarily by proliferation of basal cells and other nonciliated epithelial cells. Basal cells express p63 (Trp63) and variable patterns of cytokeratins (e.g., CK5 and CK14). Basal cells are capable of self-renewal and differentiate into ciliated, goblet, and other secretory cells in the airways in vivo and in vitro [105,108,109]. After severe lung injury, basal cells lining the airways and within the ducts of tracheal–bronchial glands migrate along the airway surfaces, proliferate and differentiate to contribute to repair the respiratory epithelium [105,110,111]. Lineage tracing studies demonstrate significant plasticity between basal cells and various subsets of nonciliated epithelial cells in the airway, with multiple cells capable of rapid migration, proliferation, and differentiation during repair of the conducting airways in animal models exposed to toxicants such as naphthalene or sulfur dioxide (SO_2) [70,105,109,112–120]. In addition, purified basal and secretory cells can reestablish a complex respiratory epithelium consisting of numerous airway cell types including ciliated cells, secretory, and goblet cells in vitro [121]. In contrast, lineage tracing experiment indicate that ciliated cells are not proliferative and do not serve a role as progenitor cells after injury to airway epithelium [122]. Together these data suggest a hierarchy of airway cells with varying stem/progenitor cell capabilities, with basal cells acting as the primary professional progenitor population and substantial facultative capacity within neuroepithelial and secretory cells. Regional differences in proliferation and progenitor cell behavior occur in NEBs near bifurcations of airways, in bronchoalveolar ducts, and along vessels and stroma. Mitotic activity is induced throughout the airways following injury, although selective anatomic regions of cytoprotection or enhanced proliferative capacity may serve unique functions during lung repair. Many of these responses, including which types of cells predominate in the regenerative response, depend upon the nature, chronicity, and severity of injury [123].

In smaller airways, much recent data has focused on a progenitor population within terminal bronchioles adjacent to the bronchoalveolar duct junction, termed bronchoalveolar stem cells (BASCs) [115,124]. BASCs were originally defined as CCSP- and pro-SPC-coexpressing cells that resist naphthalene injury and repopulate terminal bronchioles. Two independent lineage tracing experiments support the ability of the BASCs to renew and differentiate into various cell populations after injury, with alveolar injury biasing BASCs primarily to distal lineage differentiation and airway injury leading to primarily regeneration of the small airways [125,126]. BASCs are not abundant, and at present the relationship between BASCs and the more abundant cells progenitor cells of the airway (e.g., secretory and basal cells) or the alveolus (AT2 cells) is not clear. BASCs have to date not been identified in human lung, and while mice have a clear bronchoalveolar duct, human lung contains more extensive small airways and alveoli branch from respiratory bronchioles which are not present in mouse lung.

In the alveolar region of the lung, AT2 cell types contain substantial progenitor capacity and act to regenerate both ATI and AT2 cells after injury [127]. Proliferation of alveolar type 2 cells begins within days of alveolar injury, and it has been known for over 40 years that a subset of AT2 cells preferentially generates AT1 cells after injury [128,129]. Recent lineage tracing studies have demonstrated that Wnt signaling, a well-known factor in lung epithelial development, activates a subset of Wnt-responsive AT2 cells that functions as a facultative alveolar progenitor lineage. These Wnt-responsive AT2 cells, called alveolar epithelial progenitor (AEP) cells, function to maintain alveolar homeostasis and drive regeneration after influenza virus infection [50,51]. After injury, Alveolar Epithelial Progenitor (AEP) cells are preferentially recruited into the cell cycle and regenerate functional AT1 and AT2 cells. More limited evidence suggests that a separate population of distal $\alpha6\beta4$ integrin-expressing epithelial cells can also enter the cell cycle and participate in alveolar regeneration [130]. Type I cells are generally sensitive to injury and seldom proliferate though under specific circumstances can participate in limited alveolar repair [131]. After very severe alveolar injury that depletes these distal progenitors, a subset of basal cells migrate into the lung parenchyma and form a Krt5-positive epithelium [51,132–135]. These cells are driven to migrate by hypoxemia and fail to differentiate into normal alveolar cells, likely representing rapid but abnormal epithelial reconstitution rather than high fidelity alveolar regeneration. Self-renewal and differentiation of airway basal cells is influenced by Notch signaling [105,136], and aberrant Notch activity is present in reconstituted epithelium still present in distal lung scar months after injury [132–134].

Thus under physiologic conditions, repair is accomplished primarily by the proliferation of endogenous progenitor cells present in each of the compartments of the respiratory epithelium. As shown in Fig. 67.6, there is increasing evidence that these cells exist in a defined hierarchy of lung epithelial progenitors with substantial plasticity at least within each compartment. Understanding these normal repair processes will serve to inform the scarring and hyperproliferative responses seen following catastrophic or chronic injury that lead to airway epithelial metaplasia, dysplasia, and hyperplasia that are associated with lung fibrosis, remodeling, and cancer. In addition, recent single-cell RNA sequencing of tissue from idiopathic pulmonary fibrosis patients suggests abnormal differentiation of respiratory epithelial cells with expression

of both proximal and distal markers, suggesting pathological pathways may be distinct from physiological repair. Major challenges faced by the field today are to understand the relationship between these various cells and identify the conditions under which each progenitor population is activated for the most effective regenerative response. Whether distinct activities of subsets of respiratory epithelial cells contribute uniquely to normal or pathological repair processes and chronic lung diseases also remains to be clarified. True understanding of these relationships will be critical to define populations for engraftment and activation that could enable regenerative therapies for pulmonary diseases.

Role of lung microvasculature in lung repair

The alveolar microvasculature actively participates in regeneration of alveoli after acute and chronic lung injuries caused by chemicals, viral, and bacterial pathogens and various environmental irritants [95,137]. Alveolar repair and regeneration require coordinated signaling events between all cells comprising the alveolar wall, including endothelial cells, pericytes, type I and type II pneumocytes, fibroblasts, and alveolar macrophages. Circulating immune cells are also involved in alveolar repair by secreting various cytokines and chemokines that stimulate proliferation and migration of endothelial, epithelial, and stromal cells to restore the alveolar architecture after injury. Epithelial and immune cells in the site of injury produce VEGF, which has a dual role in the repair process. VEGF increases survival of endothelial cells and decreases alveolar damage after hyperoxia-induced neonatal lung injury in rodents [138]. VEGF increases neonatal lung angiogenesis by stimulating endothelial proliferation, migration, and survival via VEGF Receptor 2 (*Flk1*)-mediated activation of RAS/ERK and PI3K/AKT signaling pathways. Side effects of systemic VEGF administration have been also reported and attributed to acute increase in endothelial permeability and disruption of endothelial junctions, causing lung edema and inflammation [139,140]. Stem cell factor (SCF), which is produced by multiple cell types in the site of injury, signals through c-KIT receptor tyrosine kinase present in endothelial progenitor cells to increase cell proliferation and survival [141,142]. Systemic administration of SCF stimulates neonatal lung angiogenesis and accelerates alveolar repair after hyperoxic injury in newborn rats [143].

Vascular repair is directly coupled to alveolar morphogenesis after lung injury and during compensatory lung regrowth following partial pneumonectomy [144,145]. In response to VEGF and FGF, endothelial cells produce matrix metalloproteinase 14, which cleaves heparin-binding EGF-like growth factor and release the active EGF-like fragments. The active EGF-like fragments stimulate the proliferation of alveolar epithelial cells and BASCs during lung regeneration [144]. Microvascular endothelial cells stimulate differentiation of BASCs into Types I and epithelial cells in vitro and after subcutaneous injection [146]. This process is dependent on the production of thrombospondin-1 (TSP1) by pulmonary endothelial cells because $Tsp1^{-/-}$ endothelial cells are unable to support the differentiation of BASCs [146]. Increased expression of TSP1 at the sites on lung injury was linked to BMP4 and the calcineurin/NFATc1 signaling pathway [146].

Endothelial barrier function is critical for microvascular repair after lung injury [147]. Thrombin, bradykinin, histamine, reactive oxygen species, VEGF, TNF-α, and endotoxin increase endothelial permeability in vitro and in experimental animals (reviewed in Ref. [147]). In contrast, sphingosine-1-phosphate (S1P) and Angiopoetin-1 (Ang-1) have been shown to stabilize the endothelial barrier and decrease endothelial permeability [95,147]. Maintenance of endothelial barrier is critical to decrease lung edema and inflammation after lung injury. Endothelial proliferation in vivo requires tight endothelial barrier, which is needed to ensure vascular repair in the absence of vascular leak. Multiple transcription factors are implicated in the regulation of endothelial proliferation after lung injury, including STAT3, cMYC, E2F, and the Forkhead transcription factors FOXO1, FOXM1, and FOXF1 [1]. FOXF1 increases the expression of VE-cadherin and S1PR1, a receptor of S1P, maintaining endothelial barrier function after the injury [95], and stimulates endothelial proliferation by repressing cell cycle inhibitors *Cdkn1a* and *Cdkn2b* [145]. FOXM1, a downstream target of RAS/ERK pathway [148,149], induces endothelial proliferation by increasing the expression of cell cycle regulatory genes *Cyclin B1*, *Plk1*, and *Aurora B* [150,151]. In contrast, FOXO1 inhibits cell cycle progression by repressing cell cycle regulatory genes [152]. Altogether, these studies demonstrate that vascular repair after lung injury requires multiple signaling pathways and transcription factors to activate and execute the regeneration program.

Endothelial progenitor cells in lung repair

Endothelial progenitor cells (EPCs) consist of various cell populations capable of differentiating into mature cell types, including arterial, venal, lymphatic, and microvascular endothelial cells [81]. Among well-described EPCs are bipotential hemangioblasts and hemogenic endothelium, both of which are active in the yolk sac and dorsal aorta at early stages of embryogenesis [87,153]. During lung development, endothelial cell lineages mainly derive from lung resident EPCs, pericyte-like mesenchymal progenitors, and circulating endothelial cells, reviewed in [81]. c-KIT^{+}/$PECAM\text{-}1^{+}$/$CD45^{-}$ cells are abundant in parenchyma of embryonic and neonatal lungs [154] and

may represent the lung-resident EPCs since they are capable of self-renewal and differentiation to mature endothelial cell types [141,142]. In the adult lung, c-KIT-positive EPCs are rare and endothelial lining of blood vessels is mainly repaired by proliferation of resident endothelial cells that undergo dedifferentiation to enter the cell cycle [155]. Recent studies demonstrated that endothelial regeneration in large blood vessels requires the activation of stress response gene *Atf3* in differentiated endothelial cells adjacent to the site of injury, leading to appearance of a highly proliferative endothelial subpopulation expressing FOXM1 [156]. While endothelial repair in the adult lung is mostly driven by resident endothelial cells, bone marrow−derived circulating EPCs have only minor contribution to the repair process [155]. Bone marrow−derived EPCs have a low capacity to engraft into blood vessels, but these cells are capable of regulating vascular repair via paracrine mechanisms.

EPC-like cells were originally isolated from mononuclear cell fraction of peripheral blood [157]. These cells maintained their growth in vitro, expressed CD34, FLK1, and PECAM-1, formed endothelial-like sprouts on Matrigel, and supported angiogenesis in various ischemia models; however, these EPC-like cells were unable to maintain endothelial gene expression and were minimally proliferative [158]. Myeloid-derived EPC-like cells did not promote vascular repair through direct engraftment and replacement of injured endothelial cells but rather activated resident endothelial cells by releasing VEGF and HGF [158]. Myeloid-derived EPC-like cells did not maintain the endothelial phenotype a key cellular property used to define a progenitor cell [155]. In contrast to the myeloid-derived EPC-like cells the endothelial colony-forming cells (ECFCs), a rare cell subset isolated from peripheral blood [85], exhibited properties of endothelial progenitor cells, such as the ability to form clonal colonies in vitro, expression of endothelial cell-specific surface markers, the ability to form endothelial-like sprouts in Matrigel, and contribute to de novo vessel formation in mouse hypoxia models [85,86,159]. ECFCs were propagated in vitro without losing the progenitor properties [159].

Adult vascular endothelial cells (VESCs) have been recently identified and isolated from blood vessel endothelium using cell surface markers $CD31^+$ $CD105^+$ $SCA1^+$ $c\text{-}KIT^+$ [142]. VESCs self-renew, undergo clonal expansion in vitro and generate functional blood vessels connected to the host circulation [142]. While multiple types of endothelial progenitor cells, including VESCs, ECFCs, and lung resident EPCs, can contribute to vascular repair after injury, heterogeneity and hierarchy of lung EPCs remain unclear. Recent advances in single-cell sequencing may lead to the identification of additional populations of lung EPCs. Additional studies are needed to characterize functional properties of these cells and determine whether EPCs can be used for therapeutic purposes to stimulate lung repair after injury.

Pulmonary cell-replacement strategies for lung regeneration

Identification and expansion of progenitor cells capable of engrafting, proliferating, differentiating, and functioning after introduction into the injured lung represent an active area of research. While initial studies regarding multilineage replacement of lung and other tissues by c-Kit + positive progenitor cells was invalidated [160], cKit + progenitor cells do contribute to formation of the pulmonary vasculature, but not to the respiratory epithelium or other tissue compartments of the lung [141,161,162]. Nevertheless, a number of experiments support the ability of endogenous lung progenitors to engraft into the lung parenchyma after intratracheal instillation. For example, tracheal administration of fetal lung progenitors engrafted into the adult mouse lung but only after irradiation or chemical injury [163]. Human iPSC−derived lung cells differentiated into diverse lung epithelial cell types after transplantation into the kidney capsule of immunocompromised mice [36]. Advances in ex vivo lung perfusion and ventilation, may serve to enhance viability of lung explants, via cell-based therapy, thus increasing the availability of transplantable tissue. Ex vivo perfusion and ventilation provide a model useful in testing methods for conditioning explanted lungs and for the introduction of epithelial cells into the airways or endothelial cells in the pulmonary vasculature [164]. For example, isolated perfused lung is being used to test whether iPSCs, pulmonary progenitor cells, MSCs or MSC products enhance lung repair or regeneration during ex vivo perfusion [165]. Cells or cellular products are delivered via the trachea or via vascular perfusion to test engraftment, deliver potentially therapeutic cells, or products to condition lung explants for clinical use.

Induced pluripotent stem cells for study of treatment of pulmonary disease

Advances in reprogramming of somatic cells into iPS cells are transforming biology and medicine, enabling production of patient-specific iPS cells useful for study of the pathogenesis of disease and for cell-based therapies (Fig. 67.7) (for review Refs. [166−168]). The success of exogenous stem cell−based therapies for the lung and other organs will depend on the ability to isolate, culture, or genetically modify stem cells to correct gene mutations or to contribute to organ function. Because of their capacity for self-renewal and pluripotency, it may be possible

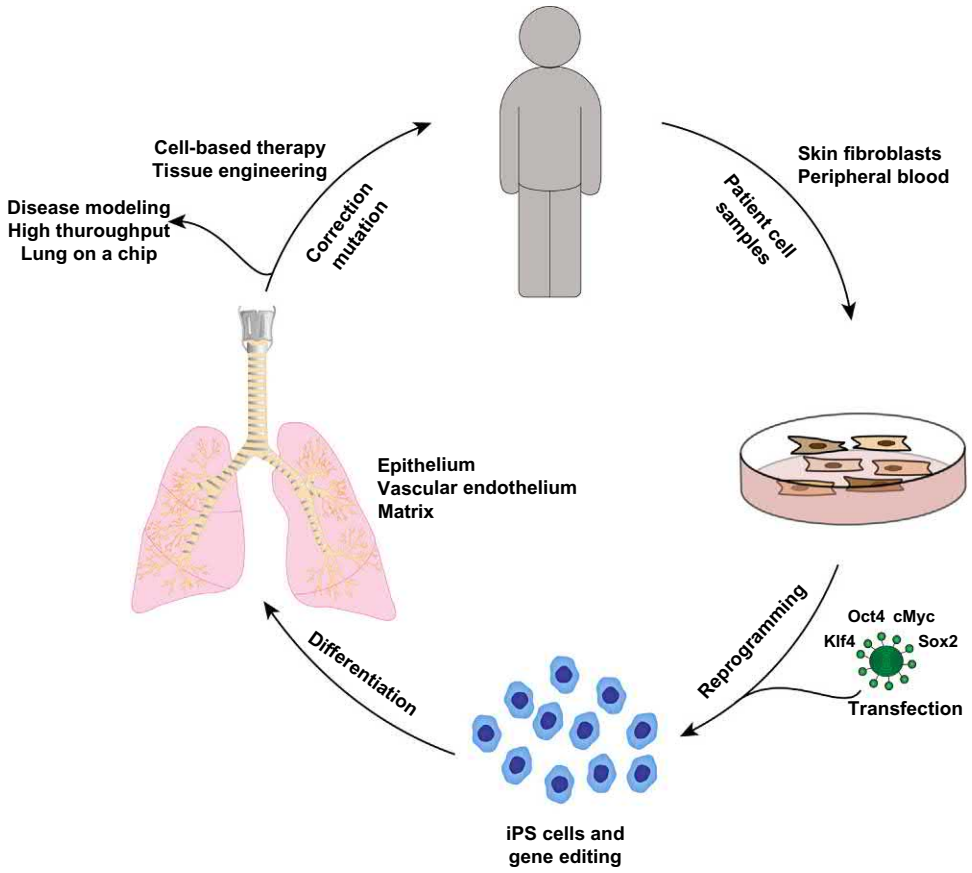

FIGURE 67.7 Induced pluripotent stem cells for study of lung regeneration. Patient fibroblasts or skin cells are grown in culture and reprogrammed by expression of transcription factors to form pluripotent stem cells. The iPSCs are cultured under conditions to induce differentiation into pulmonary endothelial and epithelial cell types. Effects of disease causing mutations on cellular functions are modeled in vitro. Mutations can be created or corrected by genetic engineering for the study of disease pathogenesis. Cells can be seeded onto biomatrices, decellularized lung scaffolds, grown in organoid cultures, and used for high throughput screening for gene and drug discovery. *iPSCs*, Induced pluripotent stem cells.

to generate large numbers of iPS cells for cell-based therapy. Reprogramming somatic cells into pluripotent stem cells overcomes social and religious barriers to the use of human ES cells. Patient-specific stem cells can be genetically modified for cell-based therapy that will substantially bypass the immunologic barriers intrinsic allograft transplantation. iPS cells are readily generated from patients' skin, blood, or other cells, and differentiated into multiple cell types in vitro, including respiratory epithelial cells. Gene transfer or genomic modification, for example, utilizing nonviral and viral vectors, TALENS, Zn-finger nucleases, and CRISPR-Cas provide the technologies enabling disease modeling and correction of genetic disorders in the future. iPS/ES cells are cultured under conditions to enhance respiratory epithelial cell differentiation. iPS/ES cells grown in defined media, at air/liquid interfaces, in organoids, or after engraftment are being used to produce a complex, highly differentiated epithelium with properties of conducting and peripheral airways. While ES/iPS cells expressing markers specific for respiratory cell types have been produced, it is unclear whether these cells fully recapitulate gene expression or other biological functions comparable to endogenous lung epithelial cells. Engineering ES/iPS cells capable of engrafting, proliferating, differentiating, and repopulating the respiratory epithelium continues to represent a considerable technical challenge.

Differentiation of induced pluripotent stem and embryonic stem cells to pulmonary epithelial cell lineages

Successful cell-based therapy for many genetic diseases affecting epithelial cell function (e.g., cystic fibrosis and mutations in genes controlling surfactant homeostasis) may require the permanent introduction of cells capable of maintaining stem cell activity as well as the ability to contribute to highly differentiate lung cell function. Differentiated respiratory epithelial cells have been produced from iPS, ES, and endogenous lung cells and expanded into organoid culture grown on various biomatrices, decellularized lung, and tracheal–bronchial grafts, in air–liquid interface cultures after instillation into the lung [163,169,170]. Cell sorting or selectable markers may be used to enrich differentiating iPS cells with lung epithelial cell characteristics during preparation. Use of cell-selection strategies and the expression of genes known to regulate differentiation of stem/progenitor cells into specific lung epithelial cell types may be used to

engineer specific cell types needed for treatment of pulmonary disorders [171]. Thus the elucidation of the signaling and transcriptional networks controlling lung cell differentiation during normal morphogenesis will be useful in guiding the production of stem cells for therapy of pulmonary diseases in the future.

Bioengineering of lung tissues

Advances in the identification and culture of endogenous lung progenitor cells and the ability to reprogram iPS or ES cells into respiratory epithelial-like progenitors enable experiments designed to seed cells into organ-like scaffolds prepared from decellularized lung tissue or on 3D-engineered scaffolds. Decellularized lung scaffolds are recellularized, reperfused, and ventilated in bioreactors for continued cell growth and differentiation [164,171]. Likewise, endothelial cells are perfused into the vascular compartment to repopulate the pulmonary microvasculature. Short-term physiological function was demonstrated after orthotopic transplantation of a recellularized lung, providing a model system with which to study lung cell biology and to test the feasibility of using various lung cell progenitor ES/iPS cells [172]. Completely synthetic scaffolds and microfluidic chambers, termed "lung on a chip," in which pulmonary cells are subjected to shear forces during ventilation and perfusion hold promise for study of lung regeneration and repair [173], Fig. 67.1.

Mesenchymal stromal cells and mesenchymal stromal cell products for the treatment of lung disease

A number of preclinical studies, primarily in rodents with infectious, chemical or ventilator induced injury, support the use of mesenchymal stem−stromal cells or their products for the treatment of acute lung injury (for review Refs. [174−176]). At present, there are approximately 80 MSC registered clinical studies designed to test their safety or efficacy for treatment of lung disease (clinicaltrials.gov). The safety of a single dose of MSCs to adult patients with ARDS was recently demonstrated [177]. MSCs are most frequently obtained from bone marrow, adipocytes, or umbilical cord blood and are being studied for treatment of various pulmonary disorders, for example, ARDS, bronchopulmonary dysplasia, lung transplant rejection, COPD, ILD, pulmonary hypertension, pulmonary cancer, and radiation pneumonitis. While early studies suggested the bone marrow cells could engraft and repopulate lung parenchyma, subsequent experiments support the concept that the cells produce paracrine factors that support tissues [178]. Conditioned media from MSCs enhanced pulmonary epithelial cell organoid proliferation and differentiation [174,179,180]. Efficacy, standardization of dose, methods of cell preparation and storage, administration, and mechanisms of action remain important issues to resolve regarding the MSC therapy for pulmonary diseases. There is increasing evidence that protective/therapeutic effects of MSCs can be conferred by exosomes, vesicles, or other products secreted by the cells. MSCs and MSC exosomes were effective in mitigating lung injury from experimental models of oxygen toxicity, ventilator, and radiation-induced lung injury in postnatal mice and are being studied for therapy of acute and chronic lung injury; for review [175,176,181−183].

Important role of the extracellular matrix in lung structure and repair

The architecture and function of the lung are dependent on the glycoprotein, collagen elastin rich extracellular matrix (ECM) that provides the scaffold upon which pulmonary cells reside and interact. Extracellular matrix supports the dynamic biomechanical forces inherent in lung ventilation and perfusion. Regenerative therapies, whether by cell or tissue replacement, will require knowledge of the multiple roles played by the ECM. Advances in imaging, proteomics, cell-matrix interactions and the use of bioreactors are providing insights into the structure and function of the pulmonary ECM or "matrisome." Pulmonary ECM is rich in elastin, collagens, glycosaminoglycans, as well as multiple signaling molecules that interact with pulmonary cells [184]. Detailed knowledge regarding composition, structure, synthesis, and remodeling of pulmonary ECM in health and disease will be needed to inform strategies to enhance lung regeneration, (for review, see Refs. [184,185]). While pulmonary scaffolds are often maintained after acute lung injury, pathological remodeling, for example, in COPD, emphysema, and pulmonary fibrosis, can be extensive; thus regenerative therapies will have to consider the abnormal tissue environments and abnormal structure of the ECM in pulmonary diseases. Successful therapies will need to repair both the abnormal cellularity, as well as the pathological scaffolds present in the diseased or injured lung. Advances in tissue engineering, for example, the use of organoids, "lung on a chip," and decellularized matrices, all represent important, recent experimental advances in the field [164,184−187]. Lung explants can be decellularized and, to remove cells of potential antigens, recellularized, with lung progenitor cells or iPSCs that are seeded onto the retained lung ECM. Procedures are being developed to reseed epithelial, endothelial, and stromal cells in ventilator-perfused explant models for study of lung regeneration. Microfluidic chambers enable coculture of

multiple cell types, recreating cell, and tissue interfaces to which biomechanical forces can be applied, for example, stretch, ventilation, and perfusion that will be useful for the study of normal and pathological pulmonary cells and tissues [188,189]. While present decellularized lung models are far from consideration for clinical application, the knowledge gained from experiments incorporating normal and pathological ECM and its recellularization will provide insights into how complex cells interact with pulmonary scaffolds to form, maintain, and repair lung structure.

Tissue engineering for conducting airways

The trachea and conducting airways are critical components of the respiratory tract whose patency and stability are required for ventilation. Airway collapse or obstruction caused by congenital malformations, tumors, or injury often requires resection and reanastomosis, surgeries that are complicated by stricture, scarring, and infection. A number of strategies have been developed, including decellularization and recellularization of bioengineered scaffolds, for tracheal repair [190]. The airways are highly complex structures whose repair is complicated by their separate bronchial circulation, abundant submucosal glands, and the complexity of the cell types and niches that comprise the trachea and bronchi. Cough and mucocilliary clearance require both stability of cartilage rings on the ventral aspect of the large airways and the flexibility of smooth muscle on the dorsal side. Stricture and scarring are common after surgical repair of the trachea or main bronchi. As such, tracheal allotransplantation has been largely unsuccessful for long segment repair of the airways. Initial encouraging reports using recellularized tracheal grafts or bioengineered tracheal grafts treated with MSCs for airway repair were not supported by subsequent clinical evaluations and were retracted from the literature [191]. Nevertheless, a variety of approaches are being studied to develop tissue and cell-based therapies for repair of tracheal–bronchial lesions, including 3D printed scaffolds which are populated with a variety of cell types [192,193].

Pulmonary macrophage transplantation for the treatment of interstitial lung disease

The pathogenesis of a number of chronic pulmonary diseases is related to defects in clearance of metabolic products from cells and tissues; for example, in hereditary storage diseases and pulmonary alveolar proteinosis (PAP). While bone marrow transplantation has been utilized to treat some of these disorders, recent experimental strategies include the correction of genetic disorders caused by defects in pulmonary macrophage function by intratracheal administration of alveolar macrophages. Hereditary PAP, a rare autosomal recessive disorder in which the lung fills with surfactant lipids and proteins, is caused by mutations in CSF2Ra and CSF2Rb (granulocyte-macrophage colony stimulating factor receptors) that impair GM-CSF signaling that controls alveolar macrophage differentiation and surfactant catabolism [194]. Mutations in the GM-CSF receptors cause pathological accumulation of surfactant proteins and lipids resulting in respiratory compromise. A single administration of normal alveolar macrophages or genetically corrected alveolar macrophage progenitors into the lungs of CSFR deficient mice, resulted in durable correction of GM-CSF signaling, restored surfactant homeostasis, and pulmonary function without the need for bone marrow transplantation [195–197]. Patient-derived bone marrow stem cells or iPSCs, which are differentiated into alveolar macrophage progenitors in vitro, are being studied for orthotopic pulmonary macrophage transplantation for the correction of hereditary PAP and other disorders of alveolar macrophage function.

Conclusion

Knowledge regarding the physiology, genetics, and cell biology of life-threatening pulmonary diseases is expanding rapidly. Likewise, progress in understanding lung morphogenesis and stem cell biology has accelerated. Together, technical and scientific advances raise hopes that novel cell and tissue-related therapies may be developed for life-threatening disorders in many organs including the lung. Major barriers to successful application of cell-based therapies for the lung remain and the ability to engineer lung tissue capable of enhancing respiratory function is presently conceptual rather than actual, Fig. 67.1. Nevertheless, rapid advances in stem cell biology, tissue engineering, and disease modeling are providing the tools that will enable discovery of the cellular and molecular processes mediating normal tissue regeneration and those needed to develop cell and tissue-based therapies for disorders affecting the respiratory tract.

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Chapter 68

Lung tissue engineering

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Introduction

The ex vivo regeneration of lung requires the simultaneous resolution of a number of complex and interlocking engineering challenges. Functional lung tissue places blood and air in close proximity to each other while allowing nearly negligible quantities of trans-vascular leak. The alveolus, the functional unit of the lung, is a delicate tissue unit made up of precise mixture of cells, extracellular matrix, and secreted proteins that allow proper ventilation and subsequent gas exchange. The air surfaces of these alveoli are coated in surfactant, a mixture of lipid and protein that gives lung tissue a tightly regulated, and unusual, response to applied mechanical stress. The epithelium of the lung is nonhomogenous, containing specialized cell classes allowing the lung to regenerate epithelial layers during normal homeostasis and to recover following grievous injury. The endothelium in the lung lines a dense microvascular network, which, together with the alveolar epithelium, maintains robust cellular junctions integral to the blood−gas barrier. Mesenchymal cells are increasingly being found to be crucial for both endothelial and epithelial support. Immune cells, meanwhile, make up nearly 60% of the cells found in lung tissue and perform important roles in both innate immunity and in the maintenance of tissue homeostasis.

Functional tissue-engineered lungs must maintain homeostatic levels of blood−gas barrier integrity, permit easy gas ventilation when transplanted into the chest (the organ must have proper compliance/elasticity properties), produce and regulate sufficient surfactant to prevent tissue damage, and contain the proper cell populations to permit appropriate postimplantation tissue homeostasis. The net result of these design criteria is that whole-lung engineering is a challenge that has, as of this writing, not been comprehensively solved. The field of pulmonary regeneration, however, is rapidly advancing. This chapter provides a broad overview of work in pulmonary regeneration to date and postulates potential advances that might be achieved in the near future as this work comes closer to clinical translation.

Design criteria for pulmonary engineering

The most basic function of the lung is to bring air and blood in close proximity to allow gas exchange. To do this, the airway tree develops adjacent to the arterial vasculature and branches into tightly packed alveoli 100−200 μm in diameter [1] which collectively resemble a closed-cell foam [2]. The walls of this foam contain a dense capillary meshwork allowing deoxygenated blood to travel from the arterioles to the venules. The tissue layer separating air from these capillaries has a mean thickness of less than half a micron [2,3], which greatly facilitates gas diffusion and allows the perfused blood to gather oxygen and to release carbon dioxide. Although this blood−gas barrier is astonishingly thin, it is nonetheless quite strong compared to the mechanics forces acting on it, with a breaking stress exceeding that of most soft tissues per unit cross-sectional area [4], and it allows nearly negligible quantities of transcapillary leak into the alveolar compartment [5]. To be translatable, any engineered lung needs to maintain near-native levels of barrier integrity and gas diffusion rates. This barrier function is the result of combined effects from endothelial and epithelial tight junctions [6−8] and from solute transport pumps actively produced and regulated by alveolar cells [9,10]. The gas diffusion rate is proportional to both the air−blood boundary surface area and barrier thickness and associated diffusion coefficient [11].

Although much pulmonary engineering has focused on regeneration of an intact blood−gas barrier, it is also

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important that the engineered construct contains well-epithelialized large airways. The large airways of the lung contain a variety of specialized mucociliary cells that are crucial in keeping the lung clear of debris and provide essential immune functions [12–14]. Most evidence suggests that tracheal and bronchial epithelial cells derive from underlying basal cells, a key homeostatic stem cell population in the pulmonary trachea and bronchi [15]. It is further important that an engineered lung have well-developed and fully endothelialized arteries and veins to allow patent blood flow to and from the capillary bed. Although the large vessels of the native organ contain mesenchymal cells key to regulating regional blood flow and pulmonary blood pressures, it is not clear if such cells are intrinsically essential for translatable pulmonary constructs. There is increasing evidence, however, that mesenchymal support of pulmonary microvessels is essential for barrier formation and regulation [16].

For transplantable constructs, it is crucial as well that the fabricated organs have the proper mechanical characteristics. A transplanted lung must be strong enough to withstand the negative pressure inflation found in the chest cavity, and compliant enough to respond to oscillatory ventilation. Further, the airway tree must be properly developed to allow ready gas flow from the oropharynx to the distal lung with very low resistance [17]. The mechanical characteristics of lung extracellular matrix are largely regulated by interstitial and mesenchymal cell populations in the lung, including fibroblasts, smooth muscle cells, and macrophages [18,19]. Perturbations to the regulation of these cell types are thought to underlie pathologies such as idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease, in which the structural mechanics of the lung are severely compromised resulting in grave tissue malfunction (reviewed in Ref. [20]).

Finally, it is likely that a truly functional engineered lung will have to generate self-sustaining levels of pulmonary surfactant. Pulmonary surfactant is an amphiphilic lipoprotein complex that greatly reduces the surface tension in the alveoli of the lung and is a major cause of the physiologic hysteresis and governing mechanical forces present in normal lung tissue [21,22]. As any implanted lung will need to be ventilated with air, the construct needs to contain and longitudinally regulates the proper surfactants to modulate the nontrivial air–fluid surface-forces present in the alveoli [23]. A summary of these most essential design criteria is schematized in Fig. 68.1.

Decellularized scaffolds and biofabrication approaches

The first studies to demonstrate the concept of whole-lung regeneration relied on decellularized cadaveric scaffolds (see Fig. 68.2). These scaffolds retain nearly all of the key histologic features of lung tissues, including complete airway morphology, fully formed vascular trees, and a patent and perfusable alveolar microvascular bed [24–26]. As these constructs are made only from extracellular matrix components and are entirely acellular, the capillary walls are highly permeable to fluid flow. This means that without cells, the constructs themselves display very low levels of barrier function, allowing the majority of arterially perfused fluid to exit the vascular compartment and enter the alveolar space [27]. Further, the blood vessel walls display bare matrix, and therefore

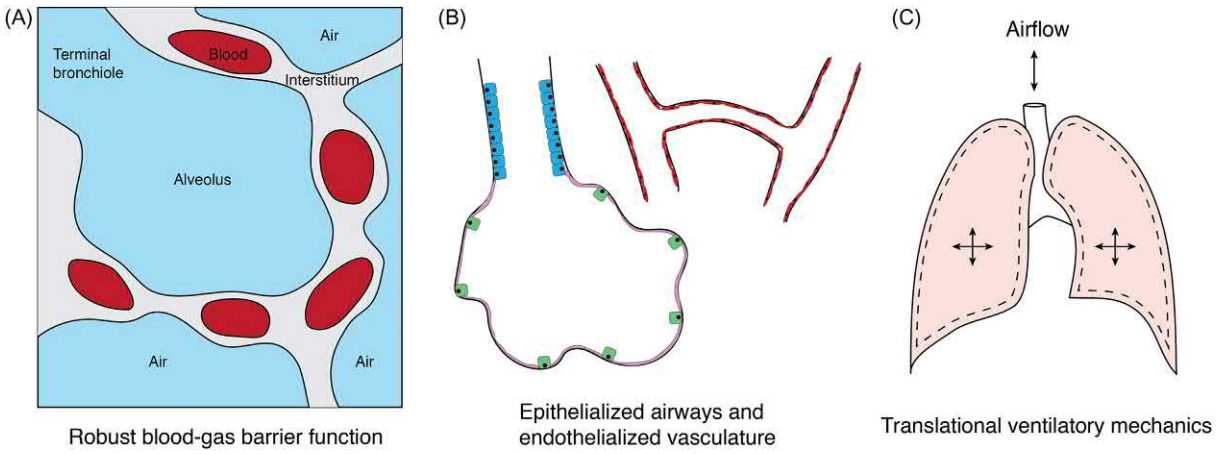

FIGURE 68.1 Essential design criteria for functional whole-organ pulmonary engineering. The primary function of the lung is to bring air and blood in close proximity to allow gas exchange. This requires a well-organized capillary–alveolar structure with robust blood–air barrier, preventing fluid leakage into the air-filled alveolus during capillary perfusion (A). Airways must be well epithelialized, and vessels must be patent and well lined with endothelium (B). Translational pulmonary constructs should have native-like mechanical characteristics to allow successful transplantation and ventilation in the chest (C).

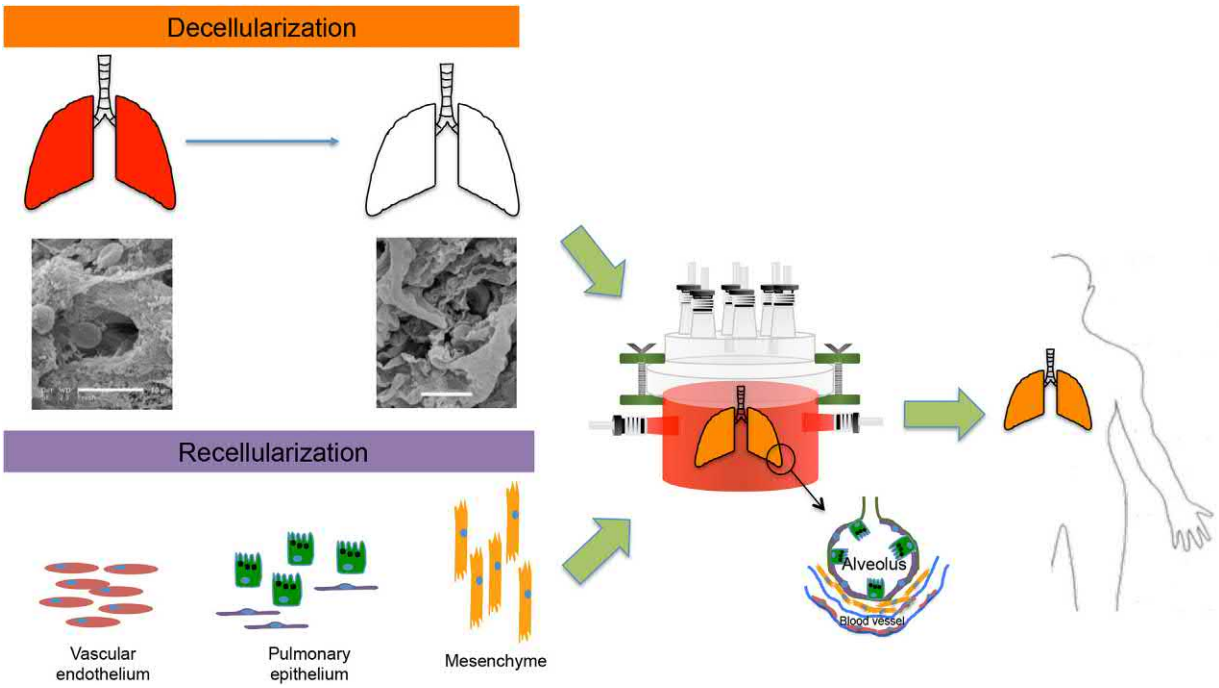

FIGURE 68.2 Schematic of whole-lung tissue engineering. The current paradigm of whole-lung tissue engineering has two separate parts: (1) Removal of cellular components from native lung tissues while preserving the intact 3D architecture and ECM components. (2) Repopulation of the decellularized scaffolds with patient-compatible cells, including vascular endothelium, pulmonary epithelium, and supporting cells such as mesenchyme to generate a bioartificial organ that provides physiological functions aiming to be an alternative source for lung transplantation.

decellularized lung is thrombogenic when implanted in vivo without sufficient endothelialization [28,29]. As there is no surfactant present, decellularized lungs are also much less compliant than native lungs when inflated with air. Decellularized scaffolds do, however, display near native levels of bulk mechanical moduli [24,30,31].

The first studies to yield decellularized lungs used a gentle perfusion of either sodium dodecyl sulfate (SDS) or 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) detergents to dissolve cellular components while retaining extracellular matrix [24,25]. Later studies found techniques based on Triton-X/SDS to be superior to CHAPS, allowing greater retention of native matrix characteristics based on quantitative proteomics [32]. In addition, it was found that decellularization at near-native pH improved the retention of matrix proteins and subsequent mechanical characteristics [33]. Although the vast majority of whole-lung studies use vascular perfusion to decellularize starting constructs, there have been some investigations into decellularization via tracheal instillation and ventilation [34,35]. In all cases, decellularized lungs present an attractive option for a starting pulmonary scaffold, as the vascular structure is preserved within the matrix and adequate mass transfer for regeneration is allowed [36]. Further, the decellularized matrix itself contains soluble and insoluble cues that drive pulmonary cell regeneration and provide spatially localized cues relevant to tissue organization and cell differentiation [37–40].

Thus far, all attempts to regenerate surgically transplantable lungs have employed decellularized whole-organ matrices. There are, however, next generation approaches on the horizon based on free-form fabrication techniques that may provide the requisite foundation to mass-produce scaffolds without the need for cadaveric donors. These scaffolds are unlikely to be fully vascularized in the near future, given the voxel resolution limits of current technologies, but can be made from biologic components retaining many desirable matrix properties and be crafted en-mass for clinically translational applications. Free-form reversible embedding of suspended hydrogels uses a gelatin support slurry to create complex three-dimensional hydrogel scaffolds [41]. This approach allows the fabrication of high-resolution complex structures from computed tomography data and is able to replicate some components of the complex branching vascular architecture necessary for whole-organ engineering with materials that promote microvascular angiogenesis [42]. Projection stereolithography is a promising ultrahigh-resolution technique capable of rapidly manufacturing complex three-dimensional structures mimicking those found in tissue extracellular architecture [43–46]. One recent and impressive topological example shows the ability to manufacture closely intertwined vascular and airway compartments [47].

Such studies clearly demonstrate that it is possible to fabricate macroscopic, perfusable constructs capable of supporting mixed cell types and performing translationally relevant gas exchange. While no study to date has shown the ability to directly manufacture constructs with the feature fineness or topological complexity of alveolar tissue, it is reasonable to predict that free-form fabrication technologies may advance sufficiently in the coming years to act as important tools in scaffold−craft for pulmonary regenerative medicine.

Pulmonary epithelial engineering

The airways and airway epithelium in the lung fulfill four major functional roles. First, the trachea, bronchi, bronchioles, and alveoli collectively form a contiguous pathway for air inhalation and exhalation, thereby enabling gas exchange with minimal applied mechanical work; second, the epithelium in the distal alveolar parenchyma is intimately involved in blood−gas barrier regulation and maintenance; without properly functioning epithelium, barrier function can be severely compromised, and fluid tends to leak into the air-filled alveolar compartment; third, pulmonary epithelium plays a crucial role in innate immunity, providing the first line of defense against inhaled pathogens; and fourth, like all epithelium in contact with the outside world, pulmonary epithelium is in a constant state of renewal, turning over in response to both grievous injury and to normal wear-and-tear. This places the epithelium in a key role in pulmonary tissue homeostasis in both the conducting and terminal airways. As epithelium in both the proximal and distal lung is nonhomogenous, there is currently significant drive to identify common progenitor cells capable of differentiating into the full variety of epithelial cells found within the native airway tree.

Proximal airway engineering

Recent single-cell studies have demonstrated that the conducting airways contain a variety of distinct, specialized epithelial cells cooperating to maintain a functional tissue layer. These cell populations include basal cells, secretory and ciliated respiratory epithelial cells, pulmonary neuroendocrine cells, and the recently profiled cystic fibrosis transmembrane conductance regulator (CFTR)-rich pulmonary ionocytes thought to be involved in the regulation of interairway ion gradients [12,13]. These cell types work together to create a robust immune barrier, secrete airway mucous, and clear inhaled particulates from the airway. There is significant evidence that basal cells act as a resident stem cell population for the proximal respiratory epithelium [48−50], with the ability to differentiate into both ciliated cells and SCGB1A1 + and MUC5AC + secretory cells [51]. This makes basal cells an attractive endogenous candidate for proximal airway regenerative engineering. Basal cells can be expanded in vitro with conditional reprogramming [52] and are thereafter capable of recapitulating tracheal epithelial morphology and phenotype when cultured on denuded or decellularized tracheas [53,54]. In studies of whole-lung recellularization, seeded basal cells successfully attach in the large airways but do not consistently recapitulate native conducting airway epithelial morphology [55], possibly due to suboptimal culture chemical and mechanical conditions during whole-organ perfusion culture.

There is some evidence that induced pluripotent stem cells (iPSCs) might be able to be used to generate translationally viable proximal airway progenitor cells. Protocols exist for generating epithelial populations expressing key proximal and distal markers, including those for basal cells; these protocols are limited, however, by their tendency to also produce contaminating mesodermal populations [56]. It should be noted, in addition, that the phenotypic regulation of regenerating epithelial populations is heavily guided by spatiotemporal cues present in both developing and native tissue, and that effective and safe protocols for generation of translational airway epithelium from iPSCs may require protocols more complex than those currently used [57].

Distal airway engineering

The alveolus of the lung has two primary cell types: alveolar type I (ATI) and alveolar type II (ATII) cells. ATII cells are large cuboidal epithelial cells that secrete pulmonary surfactant and are crucial for alveolar homeostasis [58]. ATI cells are flat, convoluted cells that make up only 7%−9% of lung cells by number but coat greater than 90% of the alveolar surface area and seal the outside of septal capillaries [59]. A single ATI cell can cover surface area in multiple alveoli, providing epithelial barrier over noncontiguous portions of the alveolar capillary network [60]. The blood−gas barrier is so thin in alveolar tissue that ATI cells and capillary endothelia generally share a common basement membrane [61]; gas exchange occurs across this very fine sandwich of ATI cell cytoplasm, matrix/interstitium, and endothelial cell cytoplasm [3]. ATII and ATI cells together are majorly responsible for the regulation of fluid balance in the alveolus and subsequently the efficacy of gas exchange [62].

ATII and ATI cells together form tight-junction networks that are integral to pulmonary blood−air barrier function [63]. The topological complexity of the pulmonary blood−gas barrier presents special challenges for reseeding and engineering efforts, considering that any seeded cells need to not only attach and be viable, but to organize their morphology and intra- and intercellular

machinery properly to recapitulate native levels of barrier hydraulic conductivity without compromising capillary flow. This is a nontrivial engineering requirement. To date, no whole-organ pulmonary engineering study has been published, which demonstrates native levels of capillary hydraulic conductivity and associated negligible trans-vascular leak.

Two primary approaches have been taken for the recapitulation of alveolar epithelium, namely, isolated or expanded primary cell seeding and the delivery of iPSC-derived populations. ATII cells have long been known to be the progenitor stem cell population for ATI cells, maintaining an injury-privileged population that sustains itself over time while reconstituting the ATI population when necessary [64,65]. Recent work has further identified a narrowly defined subpopulation of ATII cells dubbed alveolar epithelial progenitors with a distinct transcriptomic profile capable of regenerating the alveolar epithelium following injury [66]. ATII cells are therefore a promising candidate for the reconstitution of the alveolar epithelium. Early studies in lung distal airway recellularization used a mixed epithelial-enriched population isolated from fetal lungs that included both ATI and ATII cells and generally showed evidence of ATII cell engraftment and surfactant protein production [24,25,67]. A select few studies have investigated the option of engineering distal pulmonary epithelium from iPSC lineages. Ghaedi et al. showed in 2013 [68,69] that ATII- and ATI-like cells could be generated from human iPSC lineages and could successfully engraft in acellular matrices and retain key alveolar epithelial characteristics. Full characterization of and control over iPSC-derived alveolar progenitors will likely require sophisticated understanding and manipulation of genetic control modules governing the behavior of these inherently plastic cells [70–72].

Interestingly, conducting airway cells may also provide an avenue to alveolar epithelial regeneration. Studies of postinjury alveolar regeneration show that SFTPC + / SCGB1A1 + cells, labeled bronchioalveolar stem cells, are capable of regenerating epithelial layers in both the proximal and distal airways following injury [73,74]. Very recent work has suggested that proximal airway cells may even be capable of differentiating directly into ATI cells without traversing an ATII-phenotype intermediate [75]. Collectively, these studies do imply that a proximal progenitor stem cell, when coaxed appropriately, may be able to repopulate the full alveolar epithelium. Gilpin et al. showed evidence suggesting that an isolated primary basal cell population was able to colonize the distal region of decellularized lung tissue and expand to create flattened epithelial layers resembling those found in the native alveolus [55]. Such evidence supports the possibility that a common lung epithelial progenitor may either exist, or be engineered, to allow full pulmonary epithelial regeneration covering both proximal and distal populations.

Mesenchymal support of pulmonary epithelium

There is significant evidence that mesenchymal–epithelial cross talk influences epithelial phenotype. Although the first studies in whole-lung regeneration used a mixed cell type population isolated from fetal tissue, in general the focus was on epithelial and endothelial reseeding, and there is now a substantial body of literature showing that close consideration of mesenchyme and mesenchymal subtypes may be necessary to appropriately guide and regulate pulmonary epithelial regeneration in decellularized scaffolds. Both WNT and fibroblast growth factor (FGF) signaling are known to be important for lung epithelial morphogenesis during development [76]. Single-cell sequencing of the alveolus has revealed IL-6, BMP, and FGF signaling modalities to be key regulators of the ATII cell niche [77], and there is evidence that FGF10 is a key component of the basal cell niche [78]. Although these and other epithelial niche molecules can be dosed systemically to ex vivo engineered lungs, such an approach uniformly delivers soluble factors to the entire construct, which is unlikely to promote native-like histologic complexity. It is therefore preferable, and very possibly necessary, to provide appropriate cell populations to allow in-organ self-organization of the stem-cell niches necessary for normal organ homeostasis. The accurate mapping of cell niches is still in its infancy, but the advent of single-cell technologies is rapidly transforming this field and is likely to yield great insight applicable to pulmonary regeneration in the coming years.

Pulmonary endothelial engineering

Although pulmonary capillaries occupy only ~2% of lung anatomic volume, the microcirculation contributes to over 70% of gas exchange and nutrient transport to the whole parenchyma (reviewed in Ref. [79]). Endothelial cells, lining the inner surface of blood vessels, synthesize many factors, including nitric oxide, thrombomodulin, and prostacyclin, thereby regulating vascular homeostasis ([80–88] and reviewed in Ref. [89]). Dysfunction of pulmonary microvascular endothelial cells increases vascular permeability, leading to extravascular leak of protein-rich edema, polymorphonuclear leukocyte influx, microvascular thrombosis, and further lung dysfunction (e.g., acute lung injury and sepsis) [90]. In addition, microvascular endothelium plays an important role in the regulation of ATI and ATII epithelial cells, affecting both ion channel and barrier formation [91,92]. One of the key failure modes in engineered lung implantation is impaired endothelial coverage and insufficient microvascular barrier, which leads to thrombosis and

pulmonary edema, respectively [24,25]. Thus reconstruction of the pulmonary vasculature, especially the microvasculature, in decellularized lung scaffolds is of pivotal importance for maintaining pulmonary vascular homeostasis, alveolar function, and allowing complete lung regeneration.

Endothelial cell sources for lung tissue engineering

Pulmonary endothelial cells

Endothelium in lungs displays a distinct phenotype from endothelium in other organs. Lung microvascular endothelial cells (ECs) secrete markedly more urokinase-type plasminogen activator antigen than ECs from umbilical veins, angioma, or liver [93]. Nolan et al. established organ-specific molecular libraries of microvascular ECs and revealed that microvascular endothelium in lungs uniquely expresses many markers such as kit (CD117), CD36, and TBX3 as compared to ECs in brain, kidney, liver, and heart [94]. Endothelial cells are a heterogeneous population of cells not only with respect to different organs, but also among microvasculature, arterial, venous, and lymphatic systems. Pulmonary microvascular endothelium tends to uniformly bind *Griffonia simplicifolia* lectin, whereas endothelium from large vessels preferably binds to *Helix pomatia* lectin. These cells possess no Weibel–Palade bodies, less von Willebrand factor as compared to their counterparts in medium and large vessels ([95–97] and reviewed in Ref. [98]). In addition, under in vitro culture, microvascular ECs form significantly tighter barrier and proliferate faster than do ECs from large vessels [99,100]. This is supported by the fact that there is higher proportion of endothelial colony forming cells in microvasculature than in the large vessels [99]. Given the phenotypic discrepancy of endothelium in lung tissues, the ideal cell candidate for lung microvasculature engineering should be from the pulmonary microvasculature itself. We have previously repopulated decellularized lung scaffolds with pulmonary microvascular endothelium. The cells not only maintained their molecular phenotype but also displayed tight junctions after 8-day culture [24]. However, engineered lung explants in this study still showed impaired vascular barrier formation. Coculture of microvascular ECs with arterial and venous ECs in decellularized lung scaffolds increased their VE-Cadherin-based barrier function, suggesting a necessity of cell–cell cross talk for barrier function [101]. Although the field's current success is promising, it is well documented that lung allograft endothelium can initiate immune rejection through presentation of alloantigens to circulating T cells, natural killer cells, and macrophages (reviewed in Ref. [102]), which limits the use of primary pulmonary ECs for translational applications.

Induced pluripotent stem cell derived endothelial cells

iPSCs, derived from differentiated adult cells through genetic reprogramming, are not only theoretically able to generate unlimited numbers of cells but also to potentially bypass the issues of allogeneic immune rejection [103–105]. Differentiation toward endothelial cells from iPSCs usually applies growth factors, coculture with parenchymal cells, or two-dimensional culture on ECM proteins (reviewed in Refs. [106,107]). Ren et al. incorporated Wnt activation with CHIR99021, transforming growth factor (TGF)-β inhibition with SB431542 and hypoxic conditions in an 8 day differentiation protocol and achieved a homogenous population of endothelium [108]. After coculture with iPSC-perivascular cells in rat decellularized scaffolds, cell coverage reached ~75% of native counterparts, demonstrating the feasibility of using such cells for lung tissue engineering. Recent work by Prasain et al. identified a Neuropilin 1 (NRP-1) + / CD31 + /CD144 + subpopulation from iPSC-differentiated endothelial cells that maintained stable endothelial phenotype and function and did not undergo replicative senescence for 18 passages in vitro. These iPSC-NRP-1^{+}CD31^{+}ECFC showed capacity to form human vessels in mice and to repair the ischemic mouse retina and limb for >6 months with lack of teratoma formation [106,109]. Our lab went on to culture these cells in rat decellularized lung scaffolds [110]. After 5 days, H&E staining indicated native-appearing endothelial cell coverage in both large and small vessels throughout the organ, while the matrix remained intact. Interestingly, both protein and gene expression levels of PECAM1, CDH5, TEK, and ANGPT1 were higher in cells cultured in lung scaffolds as compared to cells on tissue culture plastic, indicating improved endothelial phenotype, which may be due to the influences from microenvironmental cues still present in decellularized tissues. Thus iPS-differentiated endothelium may be a viable cell source for the vascularization of engineered lung tissues. However, many hurdles remain to be overcome; first, iPSC-derived ECs are generally heterogeneous and may possess different phenotypes and functions as compared to native microvascular pulmonary ECs; second, the intrinsic qualities of self-renewal and pluripotency of iPSCs are responsible for tumorigenic potential, which is a huge safety issue for clinical applications [111]; finally, the immunogenicity from iPSCs remains controversial and requires more extensive investigations [112]. Thus iPSC-based therapies are still in their infancy, and key challenges must be overcome before their clinical application becomes a reality.

Endothelial seeding into lung scaffolds

Early studies demonstrated that endothelial cell seeding into the vasculature of decellularized lungs was possible.

However, vascular function was impaired, in terms of both barrier formation and thromboresistance, possibly due to insufficient cell seeding and improper culture conditions [24,25]. The seminal work by Ren et al. has moved the field forward by significantly improving the EC coverage in lung scaffolds [108]. They have used gravity-driven perfusion to sequentially inject cells into pulmonary artery (PA) and pulmonary vein (PV), respectively, to minimize the loss of hydrostatic pressure as the aqueous phase diffuses through the permeable basement membrane (Fig. 68.3). After coseeding with human umbilical vein endothelial cell (HUVECs) and human mesenchymal stem cells using this method, they achieved markedly higher EC coverage as compared to previous seeding strategies [108]. When seeded in a similar fashion with rat lung microvascular ECs, lungs reach ~80% cell coverage compared to native controls after 4 days of culture and are perfusable in an implantation model. However, following implantation the airspaces also contain blood, indicating the existence of significant vascular barrier leakage [29]. Although recent literature has reached a consensus that dual-side seeding from both PA and PV increases seeding efficiency [29,101,108], many other confounding factors such as the cell size, cell density, pump- versus gravity-driven perfusions, and careful monitoring of delivery pressures are crucial in the optimization of endothelial seeding techniques.

Organomimetic endothelial culture

Chemical and physical stimuli such as shear stress, substrate stiffness, oxygen tension, and growth factors in the microenvironment of pulmonary microvasculature are of vital importance for maintaining vascular homeostasis in vivo. Shear stress is the force per unit area created when a tangential force (blood flow) acts on a surface (endothelium). Endothelium lining the blood vessels is highly sensitive to hemodynamic shear stress that acts at the vessel luminal surface in the direction of blood flow (reviewed in Refs. [113,114]). Compared to cells under pathological disturbed flow, there was a downregulation of several proinflammatory cytokines, proangiogeneic factors, and proatherogenic factors in ECs under undisturbed physiological laminar flow [115]. Most recent studies, including ours, have used low arterial perfusion rates, which is markedly lower than physiological levels [116] to culture various endothelial cells in decellularized lung scaffolds [29,108,117]. Interestingly, these studies have all achieved relatively high cell coverage, suggesting high cell viability and proliferation, which may be due to sufficient nutrient transport across the entire organ. However, the wall shear stress in vasculature, especially microvasculature, is difficult to accurately model in decellularized organs due to the leaky nature of the constructs, which makes it hard to understand mechanical influence over cellular behaviors in this context.

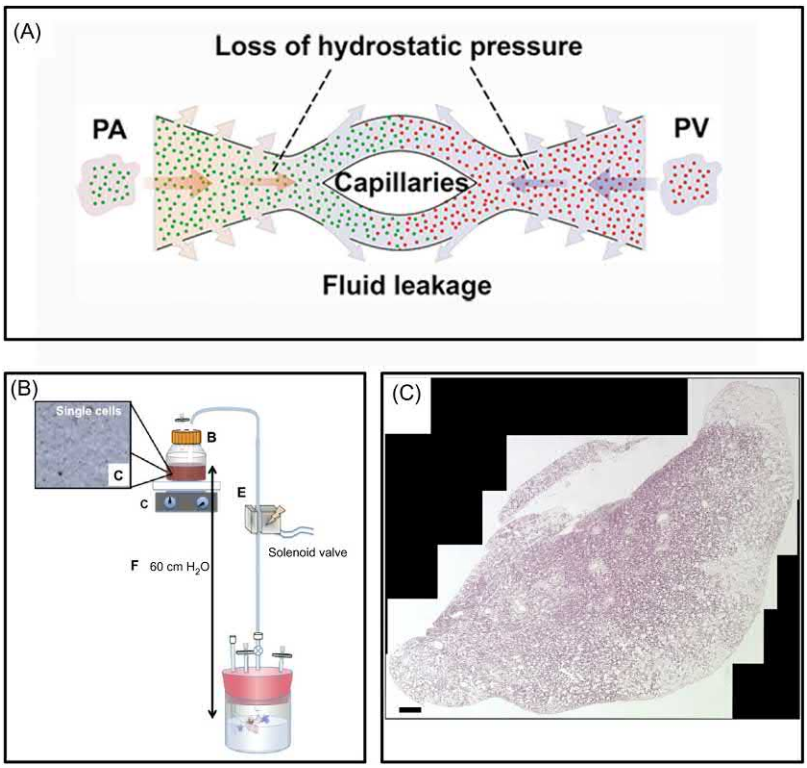

FIGURE 68.3 A diagram showing fluid flow through the PA and PV in decellularized lungs, demonstrating fluid leakage from vasculature and gradual reduction of hydrostatic pressure along vasculature (A). Bioreactor setup for RLMVECs seeding into decellularized lungs (B). EVOS image of hematoxylin and eosin staining of a lobe of decellularized rat lung seeded with RLMVECs (C). *PA*, Pulmonary artery; *PV*, pulmonary vein; *RLMVEC*, rat lung microvascular endothelial cells. *All figures have been reproduced with permission from Le AV, Hatachi G, Beloiartsev A, Ghaedi M, Engler AJ, Baevova P, et al. Efficient and functional endothelial repopulation of whole lung organ scaffolds. ACS Biomater Sci Eng 2017;3 (9):2000–10; Ren X, Moser PT, Gilpin SE, Okamoto T, Wu T, Tapias LF, et al. Engineering pulmonary vasculature in decellularized rat and human lungs. Nat Biotechnol 2015;33(10):1097–102.*

Angiogenesis describes the formation of new vessels from existing blood vessels. This process usually involves proliferation, migration, and differentiation of endothelial cells. Many growth factors can regulate pulmonary angiogenesis, including vascular endothelial growth factor (VEGF), eNOS, FGF, TGF-β, and substance P (reviewed in Refs. [118−122]). Ren et al. have cultured HUVECs-seeded lung scaffolds in "Angiogenic medium" that had high levels of serum and angiogenic growth factors such as VEGF and FGF for 14 days. However, they have not obtained increased endothelial coverage nor barrier formation compared to 1 day after seeding, which may be due to matrix degradation during angiogenesis and impaired cell stability in the scaffold [108].

During EC angiogenesis, cells are activated upon exposure to pro-angiogenic factors. These cells are prone to matrix degradation, secrete pro-inflammatory molecules, show increased intercellular permeability, and participate in vascular remodeling. Thus maintenance of EC maturation and quiescence after proliferation is critical for the stabilization of new blood vessels. Chemical factors, including protease inhibitors, sphingosine 1-phosphate, and cyclic AMP, have been discussed to induce the quiescent state of endothelial cells and can be used for vascularization in lung scaffolds reviewed in Refs. [123−130]. Our recent study investigated whether various molecules could favorably impact endothelial functionality after seeding into decellularized lung scaffolds. We compared the effect of 11 different molecules that were previously reported to improve endothelial barrier function under various platforms, and we demonstrated that Epac-selective cAMP analog 8CPT-2Me-cAMP not only improved endothelial barrier but also sustainably maintained improved barrier function for at least 3 days. After culturing in decellularized lung scaffolds, treatment with the Epac agonist significantly improved the barrier function of iPSC-ECFC-repopulated lungs for at least 6 hours [110]. This study not only demonstrates that Epac agonists may be useful to improve endothelial functionality but paves the way for applying small molecules in whole-organ tissue engineering.

Mesenchymal support of pulmonary microvasculature

Pericytes are multifunctional mural cells that wrap around capillary endothelial cells. These cells can secrete many factors, including platelet-derived growth factor B receptor-β; S1P1-endothelial differentiation sphingolipid G-protein-coupled receptor-1 (EDG1); and angiopoietin 1-tie 2 to regulate vascular maturation (reviewed in Ref. [131]). Ren et al. cocultured iPSC-differentiated ECs with perivascular cells in decellularized lung scaffolds for 6 days and obtained better endothelial integrity and stability compared to ECs alone [108]. Consistently, Doi et al. found that preseeded ASCs differentiated pericytes stabilized the endothelial cell monolayer in nascent pulmonary vessels, thereby contributing to EC survival in the regenerated lungs. The ASC-mediated stabilization of the ECs clearly reduced vascular permeability and suppressed alveolar hemorrhage in an orthotopic transplant model for up to 3 hours after extubation [132]. Thus pericytes may represent a proper cell population to promote vascular maturation for long-term culture in a decellularized lung scaffold. However, the plastic nature of pericytes and their transition to myofibroblast phenotypes may damage vascular function and induce lung fibrosis [133,134]. In addition, current coculture studies used similar seeding techniques for both pericytes and ECs. In native lungs, pericytes are located on the basolateral side of endothelium. Further research on pericyte phenotype in decellularized lung scaffolds will help one to optimize culture protocols for maintaining their function.

Bioreactor technologies for pulmonary engineering

The ex vivo culture systems required to perform whole-lung engineering are complex bioreactors designed to replicate, as closely as possible, the conditions found in the chest cavity (Fig. 68.4). The majority of whole-lung reactors strive to provide pulsatile arterial perfusion, passive venous drainage, some degree of dynamic ventilatory control, and collection and recirculation of the transpleural effluent intrinsic to decellularized lung scaffolds [24,25,135−137]. In all cases these systems have to be engineered to maintain sterility through the entirely of setup and culture and should be built to allow necessary experimental interventions. Monitoring and regulation of nutrient, waste, pH, and gas levels is tantamount for success. Levels of dissolved gases have nonnegligible effects on cell development and phenotype [138]. Mechanical ventilatory strain affects barrier integrity in a nonlinear fashion [63,139,140], while pulsatile perfusion has been shown in multiple settings to have beneficial effects on both microvascular recruitment and endothelial function in the lung [141−143]. As this is still an evolving field, lessons are continually being learned from the closely related surgical discipline of ex vivo lung perfusion, in which intact native lungs are perfused in an operating room prior to transplant (reviewed in Refs. [144,145]). One recent advance from our laboratory now allows the real-time noninvasive calculation of capillary−alveolar barrier resistance [146], and we consider the adoption of such tools to be essential for accurate estimation of whole-organ barrier function. Although few studies have

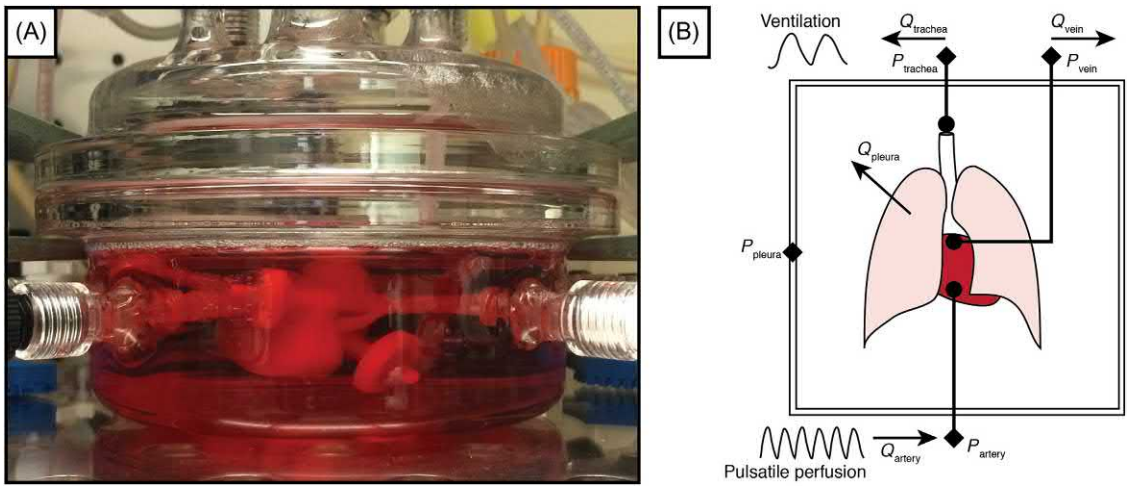

FIGURE 68.4 Cell-seeded lung scaffold cannulated and positioned within a biomimetic whole-organ lung bioreactor during organomimetic culture (A). Schematic of pulmonary bioreactor system and key culture parameters (B). Pulsatile perfusion and some degree of ventilatory stretch is generally applied. Accurate assessment of capillary–alveolar barrier function is greatly assisted by careful measurement of pressures and flows at the pulmonary artery, vein, trachea, and pleura. *(B) After Engler AJ, Raredon MSB, Le AV, Yuan Y, Oczkowicz YA, Kan EL, et al. Non-invasive and real-time measurement of microvascular barrier in intact lungs. Biomaterials 2019;217:119313.*

consistently applied negative-pressure ventilation to regenerating decellularized lungs, there is evidence suggesting that negative pleural pressure is beneficial to both vascular and airway mechanics in native whole lungs [147,148]. Due to a current dearth of data, further studies need to be performed to optimize ventilatory dynamics for engineered lung culture.

Conclusion

Whole-organ pulmonary regeneration is a nascent field barely 10 years old, which has the potential to transform the treatment of end-stage lung disease. The clinical application of this technology will require the solution of many fascinating challenges. At least four key cell types must be identified: translationally viable pulmonary epithelial progenitor populations, endothelial cells capable of recapitulating the pulmonary vasculature, mesenchymal subtypes necessary for lung stem-cell niche regulation, and immune populations integral to pulmonary tissue homeostasis. Further, any translational initiative will need to solve the problem of cell source, choosing to either isolate and propagate necessary cells from primary patient-unique tissues or derive the appropriate phenotypes from patient-specific iPSCs. Finally, although elaborate protocols exist for biomimetic ex vivo organ culture, the appropriate culture conditions for the regeneration of viable organs using decellularized scaffolds are by no means clear. Extensive work remains to be done to identify appropriate chemical milieus, mechanical conditioning, and experimental dynamics for the formation of an intact and well-organized parenchymal barrier in bioartificial lungs. This work, by its very nature, will likely require the collaboration of basic scientists, physiologists, medical practitioners, and engineers from both within and without the pulmonary disciplines.

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Part Nineteen

Skin

Chapter 69

Cutaneous epithelial stem cells

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Introduction

The epidermis consists of multiple cell types and layers. The outermost layer, called the stratum corneum, is composed of dead "corneocytes" that tightly adhere to each other to form a hydrophobic barrier that protects us against environmental insults, such as water loss and infection. The innermost layer at the base of the epidermis generates new cells that migrate to the surface while terminally differentiating, eventually forming the stratum corneum. Keratinocytes comprise the majority of epidermal cells; their main job is to produce intermediate filament proteins called keratins. The epidermis also houses melanocytes, fabricators of pigment; and Langerhans cells (LCs), sentinels against invaders that present foreign antigens to roaming T cells.

The epidermal surface is interrupted by orifices arising from skin appendages, such as the hair follicles (HFs) and sweat glands—these serve important functions in skin thermoregulation, sensation, lubrication, and protection. The epidermal basal layer is continuous with the adnexal epithelium, and basal cells from HFs and sweat ducts can move out and repopulate the epidermis in response to injury. The adnexal structures possess a greater degree of tissue complexity compared to the epidermis. For example, in contrast to the stratified squamous epithelium of the epidermis, the HF consists of at least eight different concentric layers of epithelia, which undergo degeneration and regeneration with each HF cycle.

The epidermis and HFs continuously generate new cells as old corneocytes and old hairs are sloughed off into the environment; thus their homeostasis and repair have been thought to depend on epithelial stem cells. Seminal work from many investigators has proven the existence of epithelial stem cells in the interfollicular epidermis (IFE) and the HF. Adult stem cells are quiescent in nature and are uniquely capable of both self-renewal and giving rise to differentiated progeny. In recent years, new evidence suggests that epidermal stem cells may not reside in the classic "epidermal proliferative unit" (EPU). In addition, the HF appears to contain additional stem and progenitor populations outside of the bulge. In this chapter, we will review old and new evidence to integrate historical dogma with newly emerging concepts in the evolving tale of skin regeneration.

Interfollicular epidermal stem cells

Physiological maintenance and renewal of the epidermis depends on proliferation of cells in the basal layer. Since epidermal renewal continues throughout ones' lifetime, it has been postulated that at least a portion of epidermal basal cells behave like stem cells (Fig. 69.1A for location of putative stem cells in interfollicular and follicular epidermis).

Bickenbach and Mackenzie, in pioneering work, devised "label-retaining cell" (LRC) methods for detecting quiescent cells in the epidermis [3]. Further, Morris showed that these cells retained carcinogen and possessed the quiescent characteristic of stem cells (reviewed in Ref. [4]). To test the concept that these cells give rise to all skin layers, a replication-deficient retroviral vector carrying the beta galactosidase gene was transduced with low frequency into the skin basal layer in vivo in mice [5]. Over a period of a month, discrete blue columns of cells could be visualized arising from the basal layer and progressing to the skin surface, thus supporting the existence of clonal EPUs—at least in the mouse epidermis. To further support this hypothesis, bromodeoxyuridine (BrDU) pulse-labeling of the basal layer revealed the presence of a small number of quiescent LRCs. Taken together, these data suggested that quiescent stem cells in

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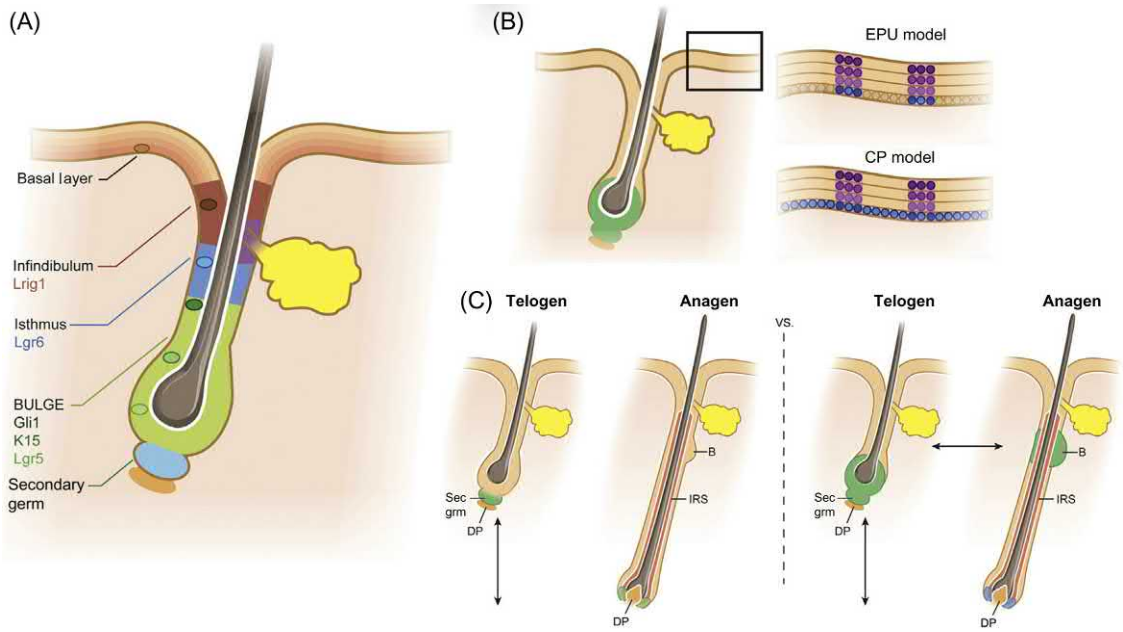

FIGURE 69.1 (A) Location of putative epidermal stem cells in the hair follicle and interfollicular epidermis. (B) Two models for interfollicular epidermal homeostasis. EPU model: in this model, rare stem cells (light blue) in the basal layer of the skin give rise to new progeny identical to itself (adjacent *dark blue cells*) or more differentiated progeny (*pink and purple cells* in upper layers). Each unit with a single central stem cell is termed an EPU. CP model: in this model, all cells in the basal layer have the same potential to make new identical progeny or more differentiated progeny. Therefore all cells in the basal layer are of the same color. (C) Two models for hair follicle regeneration during cycling. Historic model (left panel): "secondary germ cells" (named for their similarity to primary germ cells present during development) were thought to contain the stem cells for the follicle. It was thought that these cells migrate from the base of the telogen follicle to the bulb during anagen onset and then migrate back up during catagen. Current model (right panel): the secondary germ cells found at the base of the telogen follicle arise from the lowermost portion of the bulge at the end of catagen [1]. These cells migrate down into the secondary germ from their niche region where they alter their gene expression profile, proliferate, and ultimately provide all the cells for the new lower half of the anagen follicle [2]. *CP*, Committed progenitor; *EPU*, epithelial proliferative unit.

the basal layer serve to replenish the upper layers during homeostasis and following wounding. Ghazizadeh and Taichman [6] extended similar findings to human skin by using lentivirus-mediated genetic labeling of human epidermis, which showed that epidermal stem cells along the basal layer undergo an upward migration perpendicular to the epidermal surface, resembling the spatial organization of EPU.

Models for skin renewal: epidermal proliferative unit versus committed progenitor

Historically, the favored model has been that the basal layer stem cells provide "transit-amplifying" progeny that undergoes a limited number of divisions to generate the upper strata of the epidermis [7,8]. According to this model, two distinct cell populations—a slower cycling central cell and more rapidly proliferating surrounding cells—are roughly organized into a column of hexagonal unit of 10 basal cells, which lies beneath a single corneocyte (reviewed in Ref. [9], Fig. 69.1B). Based on these proliferative and morphological characteristics, the term "EPU" was coined to describe this architecture [4,8,9]. A study by Mascre et al. [10] confirmed the existence of these two described cell populations within the mouse tail IFE: a slow-cycling "stem cell" pool and a rapid cycling "progenitor" pool. Capitalizing upon the knowledge that heterogeneity in marker expression defines multiple populations within the epidermis, they followed the fate of two distinct basal populations, as defined by expression of involucrin. Their work demonstrates that involucrin-expressing basal cells divide rapidly (once per week) and quickly contribute to all layers of the epidermis, while some cells within the involucrin-negative population divide infrequently (4−6 times/year), have a distinct gene expression profile, and act as a primary contributor to reepithelization following wound repair. Work by Sada et al. [11] further supports the idea of these discrete interfollicular stem cell populations proposed by the EPU model. Utilizing GFP-tagged H2B histone (H2B-GFP) pulse-chase system in adult mouse skin, they put forward that epidermal LRCs and non-LRCs are molecularly distinct and can be differentiated by distinct genetic markings: *Dlx1-CreER* for LRCs and *Slc1a3-CreER* for non-LRCs. Long-term lineage tracing and mathematical modeling of H2B-GFP dilution data further showed that LRCs and

non-LRCs indeed constitute two distinct stem cell populations with different patterns of proliferation, differentiation, and upward cellular transport.

However, long-term lineage tracing studies have challenged the EPU-based model of epidermal regeneration. Using a low-frequency *Cre*-inducible genetic model, individual proliferating basal cells were marked and followed for 1 year in a long-term fate mapping study [12,13]. In contrast to the canonical EPU model, which predicts the size of each EPU to be finite, some epidermal clones were shown to continuously expand in size over a period of 1 year; other clones shrank and disappeared; and yet others behaved like typically expected EPUs. Mathematical modeling of these clone patterns suggested a stochastic model for epidermal renewal, in which each proliferating basal cell can give rise to two new proliferating basal cells, two differentiated progeny, or both [12]. According to this committed progenitor (CP) model, the epidermis is maintained by a uniform, equipotent population of epidermal progenitors via stochastically distributed symmetric divisions to maintain the basal layer and asymmetric divisions to generate more differentiated progeny [14]. In support of this model, data showed that a single basal epidermal cell could divide both symmetrically, producing two new basal cells, and asymmetrically, leading to more differentiated progeny [15]. Additional support for the stochastic CP model of epidermal progenitors comes from recent work by Rompolas et al., where they acquired series of temporal optical sections of the ear and paw epidermis from live H2B-GFP pulse-chased mice to track stem cell fate by position and cellular morphology [16]. From tracking cells for up to 2 weeks in the ear epidermis, they found global dilution of the H2B-GFP signal by 1 week. Furthermore, no detectable cells retained their GFP label over time, suggesting that the basal layer of the epidermis is composed of a single equipotent stem cell population that cycles at similar rates. Following the trajectory of individual cells from basal to the cornified layers above, they also found that the majority of committed cells organize into vertical columns, giving rise to the structures that encouraged the EPU hypothesis [8]. However, according to Rompolas et al., these columnar architectures would be better described as epidermal differentiation units rather than EPUs.

In reconciling the EPU and CP models, it appears that progenitor microenvironment may be the most critical regulator of stem cell fate, dynamics, and organization. Roy et al. [17] used multicolor lineage tracing with the so-called Rainbow reporter system to track individual keratinocytes in the dorsal skin epidermis of mice. Interestingly, they discovered that while IFE progenitors follow a nonhierarchical mode of development, the clones nearest to actively cycling HFs were considerably more proliferative and fast-growing than the ones more distant from the cycling HFs. This study raises a question as to whether the notion of cycling rates (slow cycling vs rapid cycling) is truly a reliable measure of evaluating "stemness" within the epidermis and suggests that spatiotemporal control of the stem cells by the microenvironment may be what is more important in regulating the potentialities of stem cells. Recent work by Liu et al. [18] suggests the differential expression of the hemidesmosome component collagen XVII (COL17A1) in individual cells guides whether a cell undergoes a symmetrical (high levels of COL17A1) or asymmetrical (low levels of COL17A1) division. Taken together, these findings suggest that the true clonal dynamics are simply too complex and condition-dependent to be reliably explained by one model, whether that is EPU or CP, and that many external factors such as the stem cell niche microenvironment may act as key regulators of stem cell potentialities.

Hair follicle stem cells

Similar to the epidermis, the HF generates a terminally differentiated keratinized end product, the hair shaft, which is eventually shed. However, in stark contrast to epidermis, the follicle undergoes a unique cyclical regenerative process known as the HF cycle [19]. As a result, the HF has a more complicated proliferative profile and architecture, with at least eight distinct epithelial lineages (Fig. 69.2). Hair is formed by rapidly proliferating matrix keratinocytes in the bulb located at the base of the growing (anagen) follicle. Interestingly, the duration of anagen varies drastically between hairs of differing lengths. For example, HFs of mouse and human eyebrow stay in anagen for only 2–4 weeks, while human scalp follicles can remain in anagen for many years. Nevertheless, matrix cells eventually stop proliferating, and hair growth ceases at catagen when the lower follicle regresses to reach a stage of rest (telogen). After telogen the lower hair-producing portion of the follicle regenerates, marking the new anagen phase (Fig. 69.2).

The bulge as stem cell source

Because the lower portion of the follicle undergoes natural cyclic bouts of rest (telogen) and growth (anagen), it was assumed that the HF stem cells (HFSCs) underlie this process. Historically, HFSCs were thought to reside exclusively in the "secondary germ," which is located at the base of the telogen HF (Fig. 69.1C). It was believed that the secondary germ moved downward to the hair bulb during anagen and provided new cells for the production of the hair. At the end of anagen the secondary germ was thought to move upward with the dermal papilla (DP) during catagen to come to rest at the base of the telogen follicle.

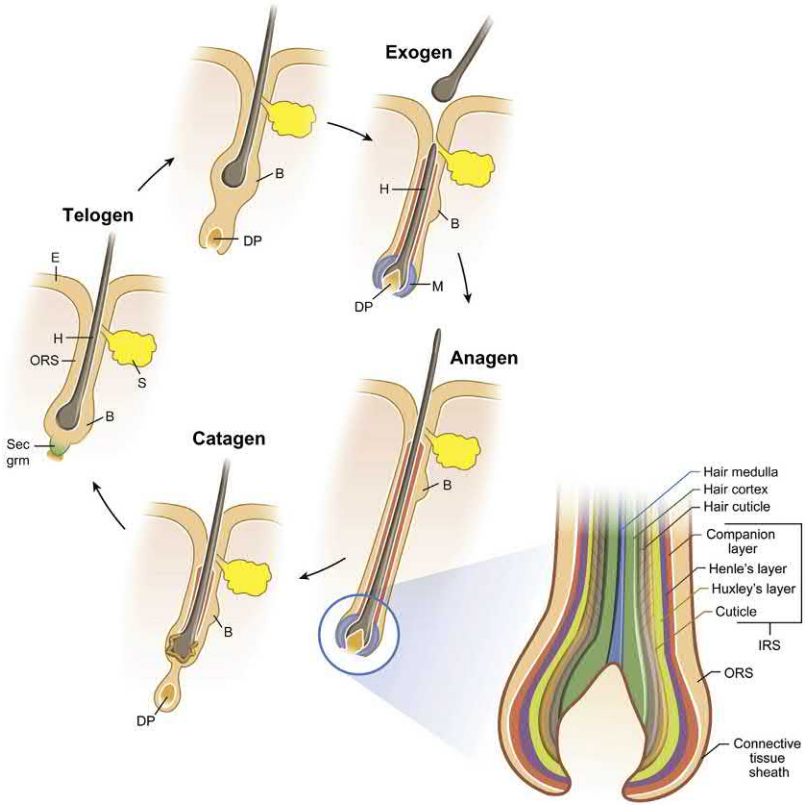

FIGURE 69.2 Hair cycle and anatomy. The hair follicle cycle consists of stages of rest (telogen), hair growth (anagen), follicle regression (catagen), and hair shedding (exogen). The entire lower epithelial structure is formed during anagen and regresses during catagen. The transient portion of the follicle consists of matrix cells in the bulb, which generate seven different cell lineages, three in the hair shaft and four in the IRS. *IRS*, Inner root sheath.

This scenario of stem cell movement during follicle cycling was brought into question when a population of long-lived cells, presumptive stem cells, was identified using LRC methods in the bulge and not the bulb [20]. Subsequent lineage studies and characterization of the bulge cells in both human and mouse follicles confirmed the bulge as the site of HFSC [20–22] (Fig. 69.3).

Defining characteristics of the bulge as a stem cell source

Multiple criteria define the bulge as the true stem cell source for HF regrowth. These include quiescence, gene expression signature, and multipotency—the ability of single cells to regenerate the entire HF in a skin reconstitution assay.

Quiescence

A salient feature of bulge cells in general is their quiescence. In both adult mouse and human skin grafted on an immunodeficient mouse, the administration of nucleoside analogs such as tritiated thymidine or BrDU, which are taken up by cells in S-phase, does not result in labeling of the bulge cells except at anagen onset [1,23]. Once labeled, bulge cells can remain labeled for 14 months in the mouse and at least 4 months (the longest period examined) in the human [21]. This prolonged quiescence is remarkable given that the surrounding cells proliferate at

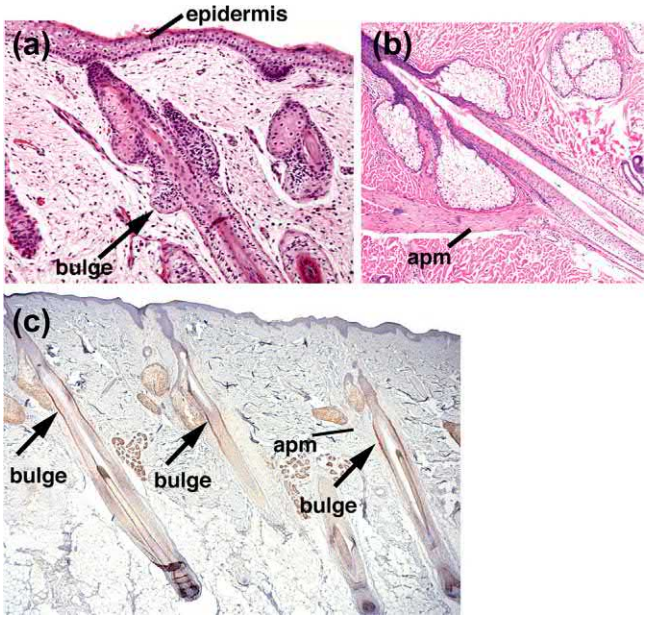

FIGURE 69.3 The bulge is a prominent structure in fetal skin (A), but generally is not morphologically distinct in the adult (B). Immunostaining for keratin 15 in scalp preferentially detects bulge cells (C). *apm*, Arrector pili muscle.

a much higher rate and further serves to suggest that bulge cells persist for the lifetime of the organism.

Several transcriptional regulators that govern bulge stem cell quiescence have been identified. Transcription factor such as nuclear factor of activated T cells c1 (NFATc1) is preferentially expressed by HFSCs and has been put forth as a key regulator involved in maintenance of bulge stem cell quiescence in all stages of the hair cycle [24]. Using immunofluorescence microscopy, the authors showed that NFATc1 exhibits a substantial overlap with CD34 and LHX2 protein expression, which are highly upregulated in quiescent bulge cells and are required for HFSC maintenance in the mouse. Another important regulator of bulge stem cell quiescence includes Forkhead box C1 (FOXC1) [25,26]. RNA sequencing (RNA-Seq) on wild-type and *Foxc1* knockout (KO) mice by Wang et al. showed that KO mice exhibited downregulation of genes associated with HFSC quiescence, including *Bmp6* and *Fgf18*. This suggests that FOXC1 is responsible for activating and maintaining quiescence gene networks in the HFSCs [25]. Lay et al. further corroborated the regulatory role of FOXC1 in HFSCs and suggested that loss of *Foxc1* lowers the threshold of HFSC activation and shortens the period in which bulge stem cells remain quiescent thereby also shortening periods between hair cycles [26].

One unique characteristic of the HFSCs is that they are able to quickly switch between quiescence and proliferation. While the precise mechanism for this is not wholly understood, recent evidence from Flores et al. [27] suggests that HFSCs maintain a metabolic state that allows them to remain dormant while still being able to quickly respond to appropriate proliferative stimuli. A transgenic mouse model with conditional alleles of lactate dehydrogenase A (*Ldha*) stopped the enzyme's production specifically in HFSCs, causing the cells to remain dormant. The LDHA enzyme is responsible for the conversion of lactate to pyruvate for energy production, thus lactate conversion seems critical for activation of HFSCs. Conversely, enhancing lactate production in HFSCs through KO of mitochondrial pyruvate carrier (*Mpc1*) resulted in accelerated activation of the hair cycle. This interesting result highlights the potential use of pharmacological disruption of lactate production to regulate the quiescence of HFSCs.

Molecular signature

Understanding the genes that distinguish bulge cells from progenitor cells in the HF and epidermis provides insights into the precisely orchestrated events of HF formation at anagen onset. Using gene expression arrays, large-scale comparisons of gene expression in bulge cells versus non-bulge basal keratinocytes [2,22] showed that genes involved in activation of the WNT pathway were generally decreased in the bulge, in line with evidence that WNT activation induces proliferation and cell differentiation and is a hallmark of anagen onset [28]. Global gene expression analyses of the human HF bulge showed many similarities to the mouse profiles, including expression of Keratin15 (KRT15), but also some important differences (Table 69.1) [31].

With the advent of single-cell RNA-Seq (sc-RNA-Seq), it has become possible to analyze the molecular signature of the bulge cells at a single-cell level. Single-cell

TABLE 69.1 Markers of stem cell subpopulations in murine and human hair follicle (HF) [bulge and hair germ (HG)].

	Murine HF stem cells (bulge)	Human HF stem cells (bulge)	Murine progenitors (HG)	Human progenitors (HG)	References
KRT15	+	+	+	+	[22,29–32]
CD34	+	–	–	+	[30,33]
NFATC1	+			+	[24,34]
LHX2	+		+		[35,36]
SOX9	+		+		[36,37]
LGR5	+		+		[38]
KRT19	+	+	+	+	[39,40]
CD200		+		+	[31,33,41,42]
GLI-1	+		+		[43]
FST		+			[31]
BerEP4				+	[44]

transcriptomics on murine telogen epidermis revealed that the bulge stem cell population segregates by distinct spatial signatures from the remainder of the epidermal cells but still shares a common basal-epidermal identity [45]. sc-RNA-Seq not only provides an unbiased, systematic way to reconstruct the transcriptional organization of the epidermis, but also it could allow for identification of previously unidentified molecular signatures that were not detectable with traditional methods such as microarrays.

Multipotency

If HFSCs are located in the bulge, then these cells should give rise to all of the epithelial cells in the lower HF. Early evidence supporting the concept that bulge cells generate the lower follicle includes proliferation studies showing that bulge cells preferentially proliferate at anagen onset [1,46]. More convincing evidence suggesting that the lower follicle originates from bulge cells came from in vivo labeling studies and transplantation studies. Taylor used a double-labeling technique to trace bulge stem cells in intact pelage follicles [47]. Faint labeling in a speckled pattern was detected in some cells of the lower follicle, suggesting that these cells had indeed originated from the bulge. Similarly, Tumbar et al. used persistence of GFP label as an indication that lower epithelial cells were progeny of the bulge cells [2]. However, neither study provided convincing evidence that all hair matrix keratinocytes in the bulb originated from bulge cells, and both suffered from inability to permanently mark bulge cells and their progeny.

Oshima et al. took a different approach and transplanted bulge regions from vibrissa follicles isolated by dissection from ROSA26 mice into non-ROSA follicles that were then grafted under the kidney capsule of an immunocompromised mouse. ROSA 26 mice express *lacZ* under the control of the ubiquitous ROSA promoter, thus the fate of the transplanted cells could be followed. After several weeks, they found labeled cells in the lower follicle, indicating that bulge cells or their progeny had migrated down the vibrissa follicle. At later time points, some follicles expressed *lacZ* in all epithelial cell layers of the lower follicle suggesting that bulge cells do generate all of the cells of the lower follicle. These elegant studies were limited by several caveats, including unclear origin of the marked cell population, the manipulation required for grafting, and the use of vibrissa follicles, which are markedly different from other mouse and human follicles.

Additional evidence for bulge cell multipotency in vivo was reported using the *Krt15* promoter to target these cells with an inducible Cre (CrePR1) construct [22]. In *Krt15-CrePR1* transgenic mice, CrePR1 remains inactive in the cytoplasm of the Krt15-positive cells except during RU486 treatment, which permits CrePR1 to enter the nucleus and catalyze recombination. Treatment of adult *Krt15-CrePR1;R26R* mice with RU486 results in permanent expression of *LacZ* in the bulge cells and their progeny. These studies showed that all epithelial cell types in the lower HF originated in the bulge.

Rompolas et al. used intravital microscopy with genetic lineage tracing at single-cell resolution to study the movement of cells throughout the follicle in live mice during HF cycling [48]. They demonstrated that the upper half of the bulge remained quiescent, whereas the lower bulge formed the new secondary hair germ (HG), which forms the new lower HF during anagen. Furthermore, they showed that when bulge HFSCs are ablated, progenitor populations above and below the bulge can directly restock the lost stem cell compartment and become bulge residents. Taken together, these results demonstrate the power of the surrounding niche for determining stem cell fate.

While the physiological roles of the observed heterogeneity and compartmentalization of HFSCs in the bulge still require further exploration, one function has been identified in the formation of tactile sensory units, which allows for relay of touch in HFs [49]. A recent report by Cheng et al. [49] showed that the epidermal stem cells in the upper bulge express a set of neurogenesis-related genes and deposit the unique protein EGF-like domain multiple 6 into the collar matrix. Meanwhile, proteins in the lower bulge remain quiescent and provide anatomically stable follicle−lanceolate complex interfaces for maintenance of robust relay of touch sensation. This unique work provides a new perspective on how sensory organs might take advantage of compartmentalized epidermal stem cell niche in order to coordinate distinct lineage tasks, all while maintaining sensory function within a structurally dynamic tissue environment.

Multiple hair follicle stem cell subpopulations by marker expression

Work from numerous labs over the last years has shown the existence of stem cell (SC) and progenitor cell populations with distinguishing marker expression:

Bulge

1. *Keratin 15 (KRT15):* KRT15 is one of the most widely used markers of bulge stem cells [21]. KRT15 mRNA and protein are reliably expressed at high levels in the bulge, but lower levels of expression can be present in the basal layers of the lower follicle outer root sheath (ORS) and IFE. Lineage tracing with a Krt15 promoter preferentially targeting the bulge stem cells showed that KRT15-targeted cells contribute to all HF

epithelial lineages [22], demonstrating its ability to serve as a powerful tool for studying bulge stem cells.

2. *Clusters of differentiation 34 (CD34)*: CD34 is a well-known marker of murine, but not human, bulge stem cells [30]. In human HFs, CD34 stains the basal cells immediately below the bulge and appear to be immediate descendants of the bulge cells. These cells are larger and more proliferative than bulge cells, thus they have characteristics of progenitor cells [33].
3. *SRY (Sex-determining region Y)-box 9 (Sox-9)*: Sox-9 is also a marker of the bulge stem cell population that is known to be one of the first genes to be expressed during HF induction in the mouse [50]. Its expression is first detected in the epithelial placode but becomes restricted to the ORS and the bulge over time. Deletion of *Sox-9* in mouse led to the development of small, atrophic hair and alopecia, illustrating the importance of Sox-9 as a functional stem cell marker that is crucial for HF homeostasis.
4. *Glioma-associated oncogene 1 (Gli1):* Zing finger protein gli1 expression defines a distinct population of stem cells in the upper bulge adjacent to perifollicular sensory nerve endings [43]. Remarkably, these nerve endings provide a niche environment by secreting sonic hedgehog protein (Shh), an essential component for the maintenance of the gli1$^+$ stem cell fate. Gli1$^+$ cells can reestablish the anagen follicle and they appear to contribute to long-term epidermal wound repair, which does not appear to be dependent upon Shh signaling (see later).
5. *Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5):* Lgr5, an R-spondin receptor implicated in canonical WNT signaling [51], marks a population of HF progenitors in the lower bulge and secondary HG during telogen, and in the lower ORS during anagen [38]. While Lgr5 expression is strongest in the secondary germ, it overlaps with the expression of KRT15. Lgr5$^+$ cells actively proliferate and contribute to all HG lineages during HF cycling [38,52]. Lgr5$^+$ cells can also reconstitute entirely new follicles when injected with dermal cells under the skin of nude mice [38]. The majority of evidence indicates that Lgr5$^+$ cells represent progenitors rather than true stem cells (see later).

Upper hair follicle

1. *Leucine-rich repeats and immunoglobulin-like domain protein 1 (Lrig1):* Lrig1$^+$ cells are actively cycling stem cells found in the junctional zone, just above the bulge and next to the sebaceous gland (SG) [53]. Lineage tracing experiments have shown that Lrig1$^+$ stem cells tend to be bipotent, physiologically contributing predominantly to infundibulum and SG lineages and only occasionally to the IFE. However, on wounding, progeny of Lrig1$^+$ stem cells have shown to be rapidly recruited to the site of injury to contribute to tissue regeneration [54]. Interestingly, mice lacking Lrig1 develop spontaneous epidermal hyperplasia and exhibit elevated levels of proproliferative cMyc protein. Further analyses indicate that Lrig1 is a downstream target of cMyc and may act to regulate cMyc expression in epidermis in a feedback loop [53].
2. *Leucine-rich repeat-containing G-protein-coupled receptor 6 (Lgr6):* Lgr6$^+$ stem cells have been reported to localize to the isthmus, immediately below the Lrig1$^+$ compartment [55]. While multipotent during embryonic development, Lgr6$^+$ stem cells undergo progressive developmental fate restriction, and in adults they participate mainly in epidermal and SG maintenance and homeostasis [55]. Interestingly, despite very close physical proximity, Gli1$^+$ stem cells in the upper bulge only marginally overlap with Lgr6$^+$ cells. In the face of trauma, Lgr6$^+$ stem cells actively contribute to closure of wounds as well as to hair neogenesis [55]. While it is a long-held belief that the Lgr6 is a marker of isthmus, a recent study has suggested that Lgr6 expression is scattered throughout the entire epithelium and may not be restricted to this particular area, warranting further research [54].

Stem cells of other ectodermal appendages

Sebaceous glands

Each HF is closely associated with the SG and, together, they constitute what is known as the pilosebaceous unit. The predominant cells in the SG, sebocytes, secrete lipid-rich products into the infundibular opening of the adjoining HF. Page et al. showed the presence of Lrig1-expressing population of stem cells within the pilosebaceous unit that are distinct from cells expressing Blimp1 [54]. By monitoring tdTomato-labeled Lrig1 progeny over time, they showed that Lrig1-expressing stem cells in the SG, initially negative for the marker Blimp1, eventually become Blimp1 positive and will renew Blimp1 + sebocytes. This result demonstrates that the Lrig1 marks a population within the SG stem cell compartment that is responsible for independent maintenance and replenishment of the SG. However, recent finding suggests that BLIMP1$^+$ cells might not be sebocyte progenitors [56]. By performing genetic lineage tracing experiments in mice, Kretzschmar et al. showed that BLIMP1-expressing cells are postmitotic and do not divide under homeostatic conditions and thus represent terminally differentiated epidermal cells within the SGs rather than a population of sebocyte progenitors.

Interestingly, cells within the SG also have been shown to be capable of dedifferentiating into stem cells in response to wounding, exhibiting a remarkable plasticity that has not yet been demonstrated in the epidermis or in other skin appendages. Using a reporter mouse model, Donati et al. demonstrated that upon IFE puncture, differentiated Gata6$^+$ sebaceous duct lineage cells in the HF duct migrate to the wound site, dedifferentiate, and start proliferating to regenerate the IFE in the wound bed as stem cells [57]. The authors also reported similar dedifferentiation of Blimp1$^+$ progenitors, hinting at the possibility that dedifferentiation may be a common attribute of terminally differentiated epidermal cells following injury.

Sweat glands

Acral skin (palms and soles in human and ventral paw in mouse) is hairless but contains eccrine sweat glands, a secretory type of ectodermal appendage that is essential for optimal thermoregulation. Eccrine glands have a relatively simple organization, consisting of a secretory glandular portion and ducts leading to the skin surface. Basal myoepithelial cells and suprabasal luminal cells comprise the glandular portion of the eccrine gland. Unlike HFs, eccrine glands are relatively quiescent. Lu et al. [58] showed that despite homeostatic quiescence, eccrine glands feature several distinct progenitor types and they exhibit regenerative potential stimulated by injury. Interestingly, sweat-producing luminal cells are maintained by suprabasal unipotent progenitors, largely independent of the basal myoepithelial cells. Neither myoepithelial nor luminal glandular cells contribute progeny toward the duct which, in turn, is maintained by its own basal unipotent progenitors. Unlike glandular cells, ductal progenitor cells become activated upon wounding and help to restore ductal openings onto the skin surface. While ductal progenitors preferentially regenerate the duct itself, they can also regenerate IFE immediately surrounding the sweat gland opening [58]. In this respect, ductal progenitors of the sweat gland share characteristics with the HF isthmus stem cells, which can also generate permanent epidermal progeny following injury [43,53,55]. Rittie showed that eccrine ductal cells also contribute to wound healing in human skin [59].

While identification of specific molecular mechanisms that underlie epithelial fate specification into different epithelial appendages is still in the works, a study by Plikus et al. [60] have highlighted the importance of bone morphogenic protein (BMP) pathway in specification of sweat gland fate decision. The study showed that overexpression of *Noggin*, a direct antagonist of BMP, in *K14-Noggin* transgenic mice results in increased HF density in body skin and transformation of sweat glands in footpads into HFs, disrupting the normal mesenchymal−epithelial BMP signaling that would have resulted in sweat gland fate specification. Similarly, a study by Lu et al. [61] identified the potential role of BMP and SHH antagonism within the epithelial bud in specifying sweat gland fate over HF fate [61]. In a genome-wide study where the back skin mesenchyme of the mouse, which is only capable of specifying to HFs, was compared to the foot skin mesenchyme, which can only make sweat glands, it was shown that the regional skin appendage specification results from differential mesenchymal expression of BMP. A strong BMP signal from the underlying mesenchyme resulted in sweat gland fate specification, whereas decreased BMP signal via SHH antagonism of BMP led to HF-specific gene expression. In fact, the authors showed that eccrine glands were converted to HF-like structures in KO mice of *Bmpr1a*, a key component of the BMP receptor kinase, demonstrating the positive role of the BMP pathway in determining glandular fate. The understanding of the role of BMP:SHH antagonism in sweat gland versus HF fate specification may help pave the path for future therapeutic advances in skin regeneration with both HFs and sweat glands.

A recent study by Yao et al. [62] has identified the transcription factor interferon regulatory factor 6 (IRF6) as another potential mediator of eccrine gland fate specification in epidermal progenitors. Using immunofluorescence, they showed that IRF6 is expressed in suprabasal epidermis in the developmental point at which eccrine gland placodes emerge, illustrating the inductive potential of IRF6 in directing sweat gland specification of epidermal progenitors [62]. Furthermore, they were able to show preferential glandular lineage differentiation in epidermal progenitor cells transfected with IRF6, demonstrating that IRF6 expression is sufficient to trigger sweat gland fate of epidermal progenitors.

Nails

The nail is a highly keratinized skin appendage covering the dorsal tip of digits, acting as an important protective covering for finger tips and toes. Just like other ectodermally derived follicular structures, the nail relies on tissue-specific stem cells to maintain homeostasis and to regenerate; however, unlike the HF bulge, the nail stem cell (NSC) niche has not yet been well-defined and its precise location still remains controversial.

The slow-cycling, label-retaining characteristics of stem cells have been used to identify quiescent reservoirs of stem cell niches in various organs, including the HF. Labeling techniques to identify in vivo LRCs have proven useful in detecting a population of nonproliferative, KRT15-positive cells organized in a ring-like cluster within the basal layer of the proximal nail fold [63]. KRT15-driven lineage tracing revealed that these cells

exhibit bipotent characteristics during normal homeostasis, in that they can contribute to both the nail structure and peri-nail epidermis, depending on the activating signals.

In contrast to the previous finding, a study by Takeo et al. [64] identified a different putative NSC niche in the proximal nail matrix using a tamoxifen-inducible lineage tracing of K14 + nail basal epidermal cells in a transgenic mouse. The lineage tracing experiment was further supported by histological analyses, which demonstrated that proximal matrix cells containing NSCs possess fewer interdigitations, a characteristic of undifferentiated epidermal cells. The Wnt pathway has important roles in hair bulge activation and HF cycling [65]. To ask if this signaling pathway was important for nail homeostasis, Takeo et al. [64] conditionally knocked out Wnt mediator ß-catenin in the epidermis of mice, and showed impaired nail growth. To ask about importance of this pathway to digit tip regeneration, they knocked out ß-catenin after tip amputation and showed that in addition to nail, bone and nerve regeneration were also impaired. The recent study by Lehoczky and Tabin [66] further confirmed the presence of this presumptive set of NSCs within the proximal nail matrix and showed that these NSCs collectively express Lgr6, an important agonist of the Wnt pathway. The dual function of Wnt signaling to induce multiple aspects of digit tip regeneration highlights the importance of this pathway that may be harnessed for new therapeutic strategies to treat amputees.

Hair follicle stem cells in skin homeostasis, wound healing, and hair regeneration

Homeostasis

In addition to the role of bulge cells in HF cycling, their contribution to the maintenance of HF homeostasis, including the IFE and SGs, has also been considered. Historically, one view was that bulge cells continuously provide progeny for repopulation of these skin regions [47,67]. However, fate mapping of bulge cells labeled in 3-week-old mice and followed for 6 months showed that the label was never observed within the IFE [22]. This indicates that $K15^+$ cells do not contribute to homeostatic maintenance of the epidermis. Furthermore, genetic ablation of $K15^+$ bulge cells does not result in IFE deficiency. Therefore these data collectively suggest that while $K15^+$ bulge stem cells can shift their fate toward the epidermal lineage, it is only in response to wound-induced signaling (see below, Ref. [68]). The other bulge populations, $Gli1^+$ and $Lgr5^+$, also appear to have no role in skin homeostasis [38,43].

Identifying the underlying mechanisms responsible for maintenance of HFs has fundamental importance in better understanding the complex process of homeostasis as well as in predicting how they will respond in face of physiological stress and injury. Choi et al. [65] proposed a key role for the Wnt/ß-catenin signaling pathway in maintenance of HF homeostasis, particularly the IFE. They showed that while short-term deletion of ß-catenin had no observable immediate effect on the $K15^+$ bulge stem cell compartment, there was a significant reduction in IFE proliferation, suggesting that IFE homeostasis and maintenance is closely regulated by Wnt/ß-catenin pathway. Another signaling pathway implicated in homeostasis, particularly in hair bulge stem cells, is the BMP/Wnt pathway [69]. By perturbing molecular signaling in the $K15^+$ stem cells, they showed that the balance between BMP and Wnt activity within each bulge stem cell is the determining factor as to whether the stem cell is activated toward HG fate (low BMP or high Wnt) or maintained in a quiescent state (high BMP, low Wnt). This suggests that the antagonistic competition between BMP and Wnt is important in balancing the stem cell activity for robust homeostasis.

Wound healing

In contrast to homeostasis, HFSCs display a remarkable degree of plasticity in response to injury. In fact, many recent studies have put forward that cellular malleability of these stem cells is what drives the repair of skin after injury [68,70,71]. Hoeck et al. [71] demonstrated that impaired hair regeneration from ablation of $Lgr5^+$ stem cells in the lower bulge is reversible, as they can be readily replaced by $CD34^+$ upper bulge cells during recovery. They showed that neighboring $CD34^+$ bulge stem cells are capable of direct conversion into $Lgr5^+$ HG stem cells, uncovering the compensatory relationship between neighboring stem cell populations that underlie regeneration from injury. Similar observation of cellular malleability has also been made outside of the bulge as well. Utilizing the *Krt15-CrePR;R26R* transgenic mouse, Ito et al. genetically marked $K15^+$ bulge cells in adult mice and showed that, in response to excisional wounding with a 4 mm (punch) trephine, the HFSCs rapidly exit the bulge and migrate upward into the neo-epidermis to participate in the reepithelization process [68]. At least 25% of the newly formed epidermis originated from these $K15^+$ bulge cells. While this transient lineage infidelity of stem cells that allows for redirection of fates is essential in wound repair and may open up new therapeutic avenues for wound treatments, a recent study suggested that a similar breakdown of stem cell lineage confinement may have an implication in cancer development [70]. This finding is in line with observations that genetically

enhanced skin stem cell activity not only closes wounds faster but also increases susceptibility to squamous cell carcinomas [72], demonstrating an intricate link between mechanisms of wound repair and the pathogenesis of cancer that still has yet to be fully explored.

Given the complexity of the wound healing process, details regarding coordination and kinetics of cellular repair as well as the effects on homeostatic functions remain largely unknown. Quantitative clonal analysis and lineage tracing of stem cells during the wound healing process revealed that the repair process does not induce a change in the balance between renewal and differentiation: the hierarchy of stem cells is maintained [73]. While the population of stem cells studied increased proliferation rates during the wound healing process, the homeostatic mode of division was conserved, leading to a linear increase in the individual clone size over time. Adding to the knowledge regarding the coordination of cellular behavior in wound repair, Park et al. [74] recently mapped the spatiotemporal dynamics of individual epithelial cells in real time following a wound. By combining intravital imaging with lineage tracing tools, they showed that the cells move out of highly proliferative zones and migrate toward the wound site, and the migration rates of these cells change depending on the distance from the wound; the migration rates are greatest closer to the wound. This coexistence of proliferation and migration in wounds impacts the stratification as well as local expansion and elongation of the repairing epithelium, contributing to its morphogenesis. Collectively, these findings all demonstrate how cellular behaviors are coordinated and orchestrated to support wound reepithelization and provide insight into how the epidermis may balance both cellular homeostasis as well as tissue regeneration in the setting of injury.

Wound-induced hair follicle neogenesis and regeneration

Harnessing the ability to grow new hair on a damaged or wounded skin is an unmet clinical need in dermatology. Pioneering work by Ito et al. demonstrated that completely new HFs can form in the center of large, full-thickness wounds via a Wnt signaling–dependent process resembling embryonic development [75]. This HF neogenesis is a relatively late event, contingent upon completion of wound reepithelialization. Neogenic HFs start as bud-like invaginations of the basal layer (*aka* HGs or placodes) and soon develop into elongated hair pegs that mature into hair shaft-producing anagen HFs within just a few days. Importantly, like normal HFs at the wound edge, these regenerated neogenic HFs have a prominent $K15^+$ bulge stem cell compartment, are able to undergo multiple HF cycles, and produce a functional hair shaft.

While wound-induced hair neogenesis serves as an illustration of adult organogenesis that is rarely observed among mammals, the molecular mechanisms and mediators that are responsible for reactivation of the observed embryonic morphogenetic programs remain poorly understood. In recent years, double-stranded DNA released from damaged skin has been identified to trigger initial skin regeneration via activation of Toll-like receptor 3, as evidenced by subsequent increases in markers of HF progenitors such as keratin 15 [76]. Epithelial expression of Msh homeobox 1 (Msx), a gene known for the induction of skin and its appendages in embryogenesis, is another potential positive mediator of wound-induced HF neogenesis (WIHN) [77]. Msx2-null mice fail to activate WIHN and successful neofolliculogenesis in wound require expression of epidermal Msx2 in two distinct temporal phases: early in the wound margin and late in the wound center [78]. Lim et al. [79] identified Shh signaling as another essential mediator for WIHN. New activation of otherwise stalled Shh signaling pathways in the wound dermis acts as a regenerative cue that overturns fibrotic Wnt-active dermal fibroblasts into a regenerative DP niche, promoting proper HF neogenesis without scarring. By demonstrating for the first time that de novo DP can be induced via modulation of Shh signaling in the dermis, the work hints at the potential of activating the Shh pathway as a therapeutic means to restore hair growth, particularly in wounded skin.

Epithelial stem cells in aging

With aging, human skin undergoes progressive architectural and physiological decline from a lifetime of different environmental insults causing damage. Age-associated degeneration of skin is usually marked by increased levels of proinflammatory mediators, decreased cell turnover, delayed wound healing, and a delayed immune response—all of which makes the skin more susceptible to breakdown and trauma [80]. However, exact mechanisms for both functional and structural decline of the skin over time are underexplored.

Recent studies suggest that some of these age-related changes are associated with loss of a hemidesmosomal transmembrane protein called type XVII collagen (COL17A1/BP180), a protein highly expressed by HFSCs for self-renewal and maintenance of their quiescence [81]. In vivo fate tracing of HFSCs revealed that HFSC aging can be triggered by neutrophil-mediated proteolysis of type XVII collagen in response to DNA damage, which eventually results in loss of stemness signatures and loss of epidermal commitment [82]. Follicle atrophy, hair loss and graying, and dysregulated hair

cycling (shortened telogen phase and prolonged anagen phase) were all observed in COL17A1 KO mice, suggesting that the type XVII collagen serves as a niche for HFSCs and plays a critical role in age-related changes to HFs.

In addition to the age-associated changes in stem cells themselves, impaired cross talk between resident immune cells and aging stem cells has been implicated in the functional decline of aging tissues, particularly with regards to delayed wound repair. Healthy murine epidermis harbors a unique population of dendritic epithelial T cells (DETCs), which promptly induce reepithelization following injury by producing keratinocyte growth factors to promote proliferation of basal progenitor keratinocytes [83]. Keyes et al. recently showed that wound edge keratinocytes in aged mice are incapable of upregulating Skints and STAT3, proteins required to activate DETCs to promote the reepithelization process following injury [84]. Notably, wound-induced reepithelization was significantly impaired in Skints KO mice, suggesting that epidermal progenitors are unable to effectively close the wound bed without the help of DETCs. While these findings do not have direct implications in humans as DETCs do not reside in the human epidermis, similar mechanisms of interaction between skin stem cells and skin-resident T cells could possibly regulate chronic wounds in aged individual.

Not all age-related skin changes are negative. In fact, dermatologists and plastic surgeons have observed that older people heal skin wounds with thinner scars. Nishiguchi et al. found that aged mice repaired back wounds with less scarring while younger mice produced more fibrotic scars, as observed from increased levels of myofibroblast marker α-smooth muscle actin [85]. In order to elucidate the mechanism mediating differential tissue repair in aged and young animals, they surgically connected the circulatory system of young and aged mice and observed that, as a result of the shared blood supply, the aged mice adopted the young mice's fibrotic wound phenotype. This intriguing work suggested that a circulating factor in the blood of young mice promotes scar formation and prevents tissue regeneration in aged mice. By comparing gene activity between injured young and aged human skin, they determined the culprit circulating factor to be stromal-derived factor 1 (SDF1), which is secreted by injured young keratinocytes to promote scar formation but is suppressed with increased activity of age-dependent EZH2. Young SDF1 KO mice indeed regenerated skin with no scarring, demonstrating that SDF1 promotes scar formation in young mice. Interestingly, SDF1 has previously been implicated in regulation of tissue regeneration not only in the skin [86] but also the liver [87]. From these findings, antagonists of SDF1 or increased expression of EZH2 may offer promising therapeutic strategies for skin scarring conditions such as keloids and hypertrophic scars.

Role of stem cells in alopecia

Compromised integrity of the stem cells in the HF bulge has been implicated in the development of alopecia. Ito et al. [68] used a transgenic mouse model to induce bulge cell destruction and demonstrated rapid and permanent loss of HFs. This work highlights the key role of the bulge cells in survival of HFs [68].

Alopecias can be classified into scarring and nonscarring types [88]; more importantly, dermatologists often further classify alopecias into temporary (reversible) or permanent (irreversible) hair loss. Recent studies suggest that the location of the perifollicular inflammatory infiltrate within the HF may explain why some types of inflammatory alopecias cause permanent follicle loss (such as lichen planopilaris and discoid lupus erythematosus), while others (such as alopecia areata) are reversible [89]. In cicatricial (scarring) alopecias, inflammation involves the superficial portion of the follicle, including the bulge area, leading to destruction of the epithelial HFSCs that are necessary for follicle regeneration. This results in permanent and irreversible loss of scalp hair. In contrast, the inflammatory injury of a nonscarring alopecia such as alopecia areata instead involves the bulbar region of the HF that is composed of bulge cell progeny. Because this area is immediately responsible for hair shaft production, its destruction leads to hair loss; however, since the bulge area is spared and remains intact, a new lower anagen follicle and subsequent hair shaft can be produced. It has been reported that patients with alopecia areata can regrow their hair, either spontaneously or in response to immunomodulation, even after having suffered from inflammation for many years.

Androgenetic alopecia (AGA, common baldness) is a genetically determined disorder that is considered to be a noninflammatory condition; however, the bulge may still be targeted for inflammation in AGA. Jaworsky et al. [90] showed that inflammatory cells localize to the bulge in patients with early AGA and postulated that this damage could contribute to the irreversible nature of AGA over time. To examine whether stem or progenitor cells are affected in AGA, Garza et al. [33] analyzed balding and nonbalding scalps from AGA patients for the presence of these cells. Fluorescence-activated cell sorting analyses showed that true noncycling $K15^+$ stem cells were retained in balding scalp, while more proliferative $Cd200^+$ progenitors residing in the lower bulge and secondary germ were markedly depleted. This result hints at insufficient stem cell activation and blunted replenishment of progenitors from bulge cells in the balding scalp of AGA, which contributes to the impaired hair

regeneration [33]. With respect to the mechanism of pathogenesis, prostaglandin D_2 (PGD_2) acts as a key inhibitor of HFSCs in AGA, and a key inhibitor of hair growth [91]. Further studies have supported this finding and showed that PGD_2 inhibits hair lengthening and HF regeneration through the G-protein-coupled receptor 44 (GPR44), highlighting the PGD_2-GPRR44 pathway as a target for treatment of AGA [91] as well as for induction of follicle neogenesis in wound healing [92].

Skin as an active immune organ

As the body's first line of defense against various toxins, pathogens, and physical stresses, the human skin is home to many immune cells. These include T cells, macrophages, and LCs; in fact, the skin harbors roughly 20 billion T cells, making the skin the largest reservoir of T cells in the body [93]. As an active immune "organ," the skin continually interacts with the circulating immune system, with resulting implications to many normal skin processes such as hair regeneration and to the pathogenesis of numerous skin diseases.

Cross talk between hair follicles and the immune system

Mammalian HFs are essential to homeostasis, regulation of body temperature, and the generation of hair shafts for protection against pathogens and physical stimuli. Apart from a protective role, mammalian HFs are a reservoir for various immune cells. Both the HF bulb and bulge are considered to be immune-privileged sites, and collapse of these areas have been associated with inflammatory disorders resulting in hair loss [94, 95], how and why the HF interacts with immune cells is still largely uncharted territory.

Ali et al. [96] found a functional link between skin-resident regulatory T cells (Tregs) and HF physiology. They showed that ablation of Tregs in mice significantly reduced anagen induction as well as successive hair regrowth, suggesting the role of Tregs in promoting the telogen-to-anagen transition. Furthermore, using immunofluorescence microscopy, they demonstrated that these skin-specific Tregs predominantly reside within 0−5 μm of the bulge region, a well-established niche for HFSCs and stimulate the proliferation and differentiation of bulge HFSCs through heightened expression of Notch ligand Jag1. Their work raises the novel possibility that immunomodulation of Tregs may be useful in devising new therapeutics for immune-mediated skin disorders such as alopecia areata.

Besides Tregs, HFs are also densely populated by unique epidermal myeloid cells known as LCs. LCs are known to provide defenses against skin pathogens such as *Candida albicans* [97] and also to help remove melanin from healthy scalp follicles during catagen [98]. But the origin of LCs in HFs is still incompletely understood. To better understand trafficking of these dendritic cells into the skin, Nagao et al. depleted LCs in Langerin-DTR (diphtheria toxin receptor) mice by injection of diphtheria and then undertook bone marrow reconstitution with CAG-eGFP bone marrow to follow eGFP-labeled LC differentiation and skin reconstitution in vivo. With induced mechanical stress, they observed infiltration and accumulation of $eGFP^+$ LCs near follicular structures, suggesting that HFs are important portals for the entry of LCs [99]. They also found that the production of CCL2 and CCL20 chemokines by HF keratinocytes is responsible for the recruitment of LCs and pre-LCs to the epidermis, further illustrating HF keratinocytes as a potent source of proteins regulating immune cell trafficking into the skin. Interestingly, they found that LCs were almost completely absent from the epidermis of scalps from subjects with lichen planopilaris, a condition characterized by inflammation and destruction of the HF bulge, corroborating the portal role of follicles and also hinting at the potential of immunomodulation as a treatment of lichen planopilaris.

Another important cutaneous immune cell population are macrophages. While the unquestionable importance of macrophages in wound healing has been known for many years, we are only beginning to elucidate their roles in HF and HFSC regeneration. Recently, Wang et al. [100] depleted $Ly6C^+$ monocytes and macrophages in mouse skin and noted delayed anagen in the wound-induced hair anagen reentry/growth (WIH-A) model, as well as in WIHN. When Ly6C + macrophages were depleted in the wound region, both WIH-A and WIHN were attenuated. This suggests that macrophages play critical roles in injury response via activation of HFSCs during anagen onset as well as HF regeneration following wounding. Subsequent microarray analysis of macrophages isolated from wound tissue also revealed tumor necrosis factor as a crucial mediator of macrophage-induced HF cycling and neogenesis in wounds.

But the role of macrophages goes beyond wound clearance and healing. In fact, macrophages have been implicated to be essential in homeostatic hair growth. Intracutaneous transplantation of bone marrow−derived macrophages into the dorsal skin of mice has been shown to strongly induce proliferation of epidermal basal cells and HF cells, including bulge stem cells, suggesting that macrophages can promote anagen [101]. Furthermore, Castellana et al. observed an apoptosis-induced decrease in the number of $F4/80^+$ $CD11b^+$ $Gr1^+$ skin-resident macrophages before the onset of anagen and showed that pathways regulating HFSC activation, such as β-catenin/Wnt signaling, are upregulated at this stage [102]. Further studies showed that

macrophage apoptosis in skin promotes premature anagen entry, possibly through release of Wnt ligands.

The inflammatory memory of skin cells

For a long time the paradigm of inflammatory memory was thought to be unique and solely restricted to innate immune cells. But recent studies have shed light on the capacity of epithelial stem cells to also document inflammatory encounters, which is notable as they frequently bear the brunt of inflammation and tissue damage. Using an in vivo mouse model of skin inflammation, Naik et al. showed that mice previously exposed to imiquimod, a Toll-like receptor-7 ligand that induces inflammation, healed the wound 2.5 times faster than controls [103]. Depletion of skin-resident B cells, T cells, and macrophages before wounding showed no effect on the healing rate postinflammation, suggesting that the epithelial stem cells are the primary cell type responsible for the observed hastened barrier restoration to the secondary challenge. The authors speculate that epithelial stem cells reprogram their chromatin and alter their accessibility in response to acute inflammatory assault. Thus upon a secondary inflammatory challenge (e.g., injury, physical stimuli), these "remodeled" stem cells rapidly upregulate transcripts governed by newly accessible chromatin domains associated with the previous stress response. A key mediator of this memory has been suggested to be inflammasome activator AIM2, which augments IL-1B to fuel the regenerative process following injury. While "inflammatory memory" by epithelial stem cells can help to quickly restore the skin barrier in response to a second breach, this heightened sensitivity to tissue damage is also likely to have many implications for skin homeostasis, cancer, and autoimmune disorders [104] such as psoriasis. Ongoing research on the inflammation-induced rewiring of epithelial stem cells is likely to provide future insights into the mechanisms of various skin diseases and bolster the development of potential therapeutic agents.

Tissue engineering with epidermal stem cells

Recent innovations in tissue engineering and biomaterials have propelled our understanding of the complex relationship between cells and their environment, allowing researchers to develop better models to study biological processes. With skin, tissue engineering with HFSCs has been implicated in the potential treatment of alopecia [105]. As a proof of concept, at least two groups have shown that freshly isolated bulge cells from adult mice, when combined with neonatal dermal cells, form HFs after injection into immunodeficient mice [22,106].

Many studies in the field looked into the self-assembling property of skin progenitor cells as a means to produce HFs from dissociated, individual cells. In fact, one of the major breakthroughs in tissue-engineered skin constructs include three-dimensional (3D) skin organoid cultures that resulted from the self-assembly of cells. By performing global gene expression analysis of human DP cells in both 2D and 3D cultures, Higgins et al. [107] discovered that there are molecular signature differences between the two environments that account for the loss of hair-inducing capacity in 2D. While assembled cells demonstrated an ability to successfully guide hair growth, this inductive property was absent from classic monolayer culture environment due to the loss of contextual and spatial signals from the neighboring epithelial cells. This demonstrates that the dermal spheroid culture better recapitulates and resembles the native, physiological tissue organization than the traditional 2D culture models, making it a potential model for applications in drug screening.

Considering the potential uses in drug screening and disease modeling, there have been a number of studies dedicated to building in vitro 3D culture systems that better capture the complex and delicate cellular cross talks of living tissue. These systems were built from mouse pluripotent stem cells [108] and even from dissociated neonatal human keratinocytes and dermal cells [109]. Weber et al. produced human hair peg−like structures in vitro from a 3D coculture of dissociated neonatal human keratinocytes and dermal cells. These cells self-organized through discrete stages, akin to early follicle development, to reach cellular configurations similar to hair pegs in situ, suggesting that these 3D culture systems can potentially provide a platform to study the spatiotemporal patterning and self-assembling behavior of skin progenitor cells. Time-lapse imaging from 3D cultures of dissociated mouse epidermal and dermal cells showed a progression through a series of morphological phase transitions, ultimately achieving a planar layer of hair primordia-bearing organoids, which resembled functional follicles: dissociated cells, cell aggregates, polarized cysts, cyst coalescence, planar skin, and hair-bearing skin [110]. Transcriptome profiling of the developing organoids revealed successive expression of distinct molecules, including growth factors and matrix metalloproteinases throughout the morphogenesis, suggesting not only that the self-assembly processes can be reinstated via environmental rewiring, but also that opportune supply of these critical regulatory molecules may be the key to successful hair formation in adult skin cells. This hints at the potential of in vitro culture systems to elucidate ways to restore and optimize HF regeneration as well as to support the production of complete HFs for possible transplantation.

Besides relying on the self-organizing ability of skin progenitor cells, there has been tremendous interest in

using bio-printed scaffolds to direct formation of HFs. For example, Abaci et al. recently demonstrated the use of 3D printing technology to incorporate HFs into human skin constructs (HSCs) [111]. By using a 3D-printed mold, they generated HF-like microwells on collagen gel in which they seeded distinct dermal cell types (fibroblasts, DP cells) in a way that recapitulated the 3D physiological conformation of cells in the native HF microenvironment. By employing this biomimetic approach, they were able to demonstrate, for the first time, the generation of human HFs in HSCs in an entirely ex vivo context. This 3D printing approach has several advantages over previously described methods that rely on spontaneous formation of HFs via self-assembly. 3D printing technology allows for tight control of spatial pattern and arrangement of cells, including HSCs, which is now known to be important for proper morphogenesis of progenitor cells into a functional follicle. Furthermore, printing density can be fully controlled, which may be advantageous in recreating the variations in hair density at the site of transplantation—something which can be personalized in transplants depending on the patient's need.

Epidermal stem cells as a therapy: the future

In recent years, combinatorial therapeutics using cultured epidermal autografts with gene therapy have emerged as a promising new concept for treating skin defects. Seminal work by Hirsch et al. [112] demonstrated that keratinocyte stem cells could be used to treat junctional epidermolysis bullosa (JEB), a severe, life-threatening congenital skin disease that is currently considered incurable. They performed an autologous skin biopsy of the patient from a nonblistering area and transduced the obtained primary keratinocytes with a retroviral vector expressing LAMB3, which is one of the mutations responsible for causing JEB. Using the cultured transgenic epidermal sheets, they were able to perform a whole-body epidermal replacement for the patient. A series of biopsies taken after grafting demonstrated successful regeneration of the entire epidermis by autologous transgenic epidermal culture and, most importantly, the epidermis had normal morphology with no sign of blisters or epidermal detachments that are hallmarks of JEB. Interestingly, clonal tracing by PCR showed that the regenerated epidermis postgraft is sustained and replenished not by equipotent progenitors but by a limited number of long-lived, self-renewing stem cells, detected as holoclones. This finding of holoclone niche supports the canonical EPU model of skin renewal [10] and suggests important implications in wound healing regarding the importance of having an adequate number of graft holoclones. While this was a single-case clinical intervention and warrants further study, these findings collectively underscore the clinical potential of genetically engineered and corrected cultured epidermal stem cells to treat wide variety of skin diseases.

Conclusion

Epithelial stem cells make essential contributions to maintenance and repair in both skin and hair. Balanced contributions by both interfollicular stem cells and more differentiated progenitors provide a constant supply of new cells during normal skin turnover and homeostasis. Interfollicular stem cells are also the primary source for new epidermis in wound healing. The HF cycles throughout the life of the individual. New work suggests that HF bulges are the likely source of true stem cells, giving rise to numerous progenitors throughout the follicle, each with distinct roles. As a mini-organ of the skin, HF macroenvironments have shown to harbor many types of immune cells, all of which are important and essential for homeostatic maintenance and regeneration after an injury. With the advent of tissue engineering, stem cells have been demonstrated as having therapeutic potential: we have seen uses in chronic wound repair, transplantation of engineered human hair, or in genetically corrected autologous epidermal sheets. Skin conditions that are considered incurable as of now may see new treatment innovations in the not-so-distant future.

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Chapter 70

Wound repair: basic biology to tissue engineering

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Introduction

The skin is the largest organ in the body whose primary function is to serve as a protective barrier against the environment. Other important functions of the skin include fluid homeostasis, thermoregulation, immune surveillance, sensory detection, and self-healing. Loss of the integrity the skin due to injury or illness compromises its protective function and when the loss is extensive may result in significant disability or even death. It is estimated that in 1996, there were 35.2 million cases of significant skin loss (US figures) that required major therapeutic intervention [1]. Of these, approximately 5 million wounds become chronic.

The most common single cause of significant skin loss is thermal injury, which accounts for an estimated 1 million emergency department visits per year [2]. Other causes of skin loss include trauma and chronic ulcerations secondary to diabetes mellitus, pressure and venous stasis. Every year in the United States there are approximately 2 million cases of chronic diabetic ulcers, many of which eventually necessitate amputation. Pressure and leg ulcers, including venous ulcers, affect another 3 million people in the United States with treatment costs as high as $8 billion annually [3]. In 2011 a survey estimated the US market for advanced wound care products, including biological and synthetic dressings, approximately $3 billion and expected to increase significantly as the population ages, becoming more susceptible to underlying causes of chronic wounds [4]. The quality of life tolls of chronic wounds are extremely high.

Over the past two decades, extraordinary advances in cellular and molecular biology have greatly expanded our comprehension of the basic biological processes involved in acute wound healing and the pathobiology of chronic wounds [5,6]. One recombinant growth factor, platelet-derived growth factor (PDGF)-BB (Regranex, Ortho-McNeil), and several skin substitutes (e.g., *dermal substitutes*: Integra Matrix Wound Dressings, Integra Life Sciences; AlloDerm, LifeCell; OASIS Wound Matrix, Healthpoint; Dermagraft and TransCyte, Advanced BioHealing; and *Epidermal/dermal substitutes*: Apligraf, Organogenesis; Orcel, Forticell Bioscience; TissueTech, Fidia Advanced Biopolymers) have reached the market place for second-line therapy of recalcitrant ulcers [7]. These therapeutic interventions have added to the clinician ability to induce skin healing, but they have not had the impact that was predicted. Regardless of the advanced wound care product, the ideal goal would be to regenerate tissues where both the structural and functional properties of the wounded tissue are restored to the levels prior to injury.

In contrast to adult wounds, embryonic wounds undergo complete regeneration, terminating in a scarless repair [8,9]. Thus investigators are now using morphogenetic cues including hair development to develop engineered constructs capable of tissue regeneration [10,11]. Cellular response to biological stimuli depends on the mechanical strength of the extracellular matrix (ECM) [12]. Therefore the therapeutic successes of tissue-engineered constructs will depend not only on their molecular and cellular activity but also on their optimal mechanical properties [13]. This chapter will begin with an overview of the basic biology of wound repair, followed by a discussion of established practices and novel approaches to engineer tissue constructs for effective wound repair. A more extensive review of wound healing at a cellular level has been published recently [14].

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Basic biology of wound repair

Wound repair is not a simple linear process in which growth factors released by phylogistic events activate parenchymal cell proliferation and migration but is rather an integration of dynamic interactive processes involving soluble mediators, formed blood elements, ECM, and parenchymal cells. Unencumbered, these wound repair processes follow a specific-time sequence and can be temporally categorized into three major groups: inflammation, tissue formation, and tissue remodeling. The three phases of wound repair, however, are not mutually exclusive but rather overlapping in time.

Inflammation

Severe tissue injury causes blood vessel disruption with concomitant extravasation of blood constituents. Blood coagulation and platelet aggregation generate a fibrin-rich clot that plugs severed vessels and fills any discontinuity in the wounded tissue. While the blood clot within vessel lumen reestablishes homeostasis, the clot within wound space acts as a growth factor reservoir and provides a provisional matrix for cell migration.

The primary cell types involved in the overall process of inflammation are platelets, neutrophils, and monocytes. Upon injury, successful reestablishment of homeostasis depends on platelet adhesion to interstitial connective tissue, which leads to their aggregation, coagulation and activation. Activated platelets release several adhesive proteins to facilitate their aggregation, chemotactic factors for blood leukocytes, and multiple growth factors [5,6], to promote new tissue formation.

Of the two primary phagocytic leukocytes, namely, neutrophils and monocytes, neutrophils arrive first in large numbers due to their abundance in circulation. Infiltrating neutrophils cleanse the wounded area of foreign particles, including bacteria. If excessive microorganisms or indigestible particles have lodged in the wound site, neutrophils will probably cause further tissue damage as they attempt to clear these contaminants through the release of enzymes and toxic oxygen products. When particle clearance has been completed, generation of granulocyte chemoattractants ceases and the remaining neutrophils become effete.

Transition from inflammation to repair

Macrophages are responsible for many of the tissue-level changes that occur as the wound progresses from inflammation to repair, as they serve important functions in both stages. It was previously held that blood monocytes would infiltrate the wound 24–48 hours after injury, during which time neutrophils accumulate in the wound. However, recent evidence suggests that monocytes can enter the wound through microhemorrhages in the wound bed within a few hours [15]. Once there, monocyte undergo phenotypic differentiation into macrophages. Macrophages exhibit a spectrum of functional phenotypes, but they are generally categorized into one of three groups: M1 or classical macrophages, M2 or nonclassical macrophages, and intermediate macrophages [16].

The current understanding of how macrophages regulate wound repair revolves around their M1 and M2 phenotypes. M1 macrophages phagocytose microbial invaders, cellular debris, and neutrophils. They aid in preventing infection and keeping the neutrophil population from overrunning the wound and causing more damage. M1 macrophages also release proinflammatory cytokines, fueling the inflammatory stage of wound repair. In contrast, M2 macrophages release growth factors to stimulate proliferation and antiinflammatory mediators to control the inflammatory response. Yet M2 macrophages still partake in the phagocytosis of neutrophils [17].

The current model of dermal wound healing identifies an "M1–M2 switch," in which the most abundant macrophage phenotype in the wound changes from M1 to M2, in accordance with the change from inflammation to repair. Evidence suggests that the microenvironment plays an important role in determining the phenotype of a macrophage. Danger-associated molecular patterns such as adenosine triphosphate (ATP) and pathogen-associated molecular patterns such as bacterial-essential polynucleotides cause monocytes to follow classical activation and become proinflammatory [15,17]. M2 activation is triggered by interleukin (IL) 3 and IL-13 instead [17]. It is not yet clear if these M2 macrophages differentiate from nonactivated monocytes or previously activated M1 macrophages [16].

Reepithelialization

Reepithelialization of a wound begins within hours after injury by the movement of epithelial cells from the surrounding epidermis over the denuded surface. Rapid reestablishment of the epidermal surface and its permeability barrier prevents excessive water loss and time of exposure to bacterial infections, which decreases the morbidity and mortality of patients who have lost a substantial amount of skin surface. If a wide expanse of the epidermis is lost, epidermal cells regenerate initially from stem cells in pilosebaceous follicles [18] but ultimately require interfollicular stem cells for completely competent regeneration of the epidermis [19]. Migrating epithelial cells markedly alter their phenotype by retracting their intracellular filaments, dissolving most of their desmosomes and forming peripheral actin filaments, which facilitate cell movement [20]. These migrating cells also undergo the dissolution

of their hemidesmosomal links between the epidermis and the dermis. All these phenotypic alterations provide epithelial cells with the needed lateral mobility for migration over the wound site. Migrating epidermal cells possess a unique phenotype that is distinct from both the terminally differentiated keratinocytes of normal (stratified) epidermis and the basal cells of stratified epidermis. It is now appreciated that the signals that control wound healing in the adult animal are similar to those that control epithelial fusion during embryogenesis [21]. A more comprehensive review of stem cells in reepithelialization has been recently published [22].

If the basement membrane is destroyed by injury, epidermal cells migrate over a provisional matrix of fibronectin, tenascin, and vitronectin, as well as stromal type I collagen and laminin [23,24]. Wound keratinocytes express cell surface receptors for fibronectin, tenascin, vitronectin, and laminin, which belong to the integrin superfamily. Integrins are distinguished by the α and β subunits (Table 70.1) [25]. In addition, α2β1 collagen receptors, which are normally disposed along the lateral sides of basal keratinocytes, redistribute to the basal membrane of wound keratinocytes as they come in contact with type I collagen fibers of the dermis. Although β1 integrins are clearly essential for normal reepithelialization [26], it is not clear which subtype is essential. It is most likely that there is a redundancy in the requirement for α5β1 and α2β1 in reepithelialization.

The migrating wound epidermis does not simply transit over a wound eschar but rather dissects through the wound, separating the fibrin/fibronectin-rich clot and desiccated dermis containing denatured collagen (together termed eschar) from underlying viable tissue [27]. The path of dissection appears to be determined by the array of integrins expressed on the migrating epidermal cells. Keratinocytes do not express αvβ3, the integrin receptor for fibrinogen/fibrin and denatured collagen [27]. Thus keratinocytes do not have the capacity to interact with fibrinogen/fibrin or denatured collagen. Furthermore, either fibrinogen or fibrin inhibits epidermal cell interactions with fibronectin, hence the migrating wound epidermis avoids the fibrin/fibronectin-rich clot but rather migrates along the type I collagen-rich wound edge via the α2β1 collagen receptor until it meets the fibronectin-rich granulation tissue and then proceeds to migrate over the newly forming tissue via fibronectin receptors α5β1 and αvβ5 (Fig. 70.1, from Ref. [27]).

ECM degradation is clearly required for the dissection of migrating wound epidermis between the collagenous dermis and the fibrin eschar and probably depends on epidermal cell production of collagenase, plasminogen activator, and stromelysin. Plasminogen activator activates

TABLE 70.1 Integrin superfamily.

Integrins	Ligand	Integrins	Ligand
β1 Integrins		αv Integrins	
α1β1	Fibrillar collagen, laminin-1	αvβ1	Fibronectin (RGD), vitronectin
α2β1	Fibrillar collagen, laminin-1	αvβ3	Vitronectin (RGD), fibronectin, fibrinogen, von
α3β1	Fibronectin (RGD), laminin-5, entactin, denatured collagens		Willebrand factor, thrombospondin, denatured collagen
α4β1	Fibronectin (LEDV), VCAM-1	αvβ5	Fibronectin (RGD), vitronectin
α5β1	Fibronectin (RGD + PHSRN, the synergy site)	αvβ6	Fibronectin, tenascin
α6β1	Laminin		
α7β1	Laminin	β2 Integrins	
α8β1	Fibronectin, vitronectin	αMβ2	ICAM-1, iC3b, fibrinogen, factor X
α9β1	Tenasin	αLβ2	ICAM-1, -2, and -3
		αXβ2	IC3b, fibrinogen
Other ECM integrins			
αIIbβ3	Same as αvβ3		
α6β4	Laminin		

ECM, extracellular matrix; *RGD*, arginine–glycine–asparatic acid.

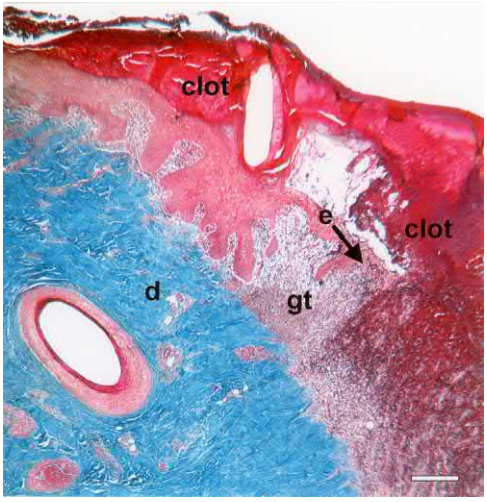

FIGURE 70.1 Histology of reepithelializing porcine wound. Full-thickness paravertebral skin wound was harvested on day 5, bisected, formalin fixed, and stained with Masson trichrome. The arrow resides on the tip of the migrating epidermis and points in the direction of migration. The eschar, which contains both clot and desiccated dermis, is denoted by "clot," normal dermis at the wound edge is indicated by the letter (d) and newly forming granulation tissue is noted by (gt). *From Kubo M, et al. Fibrinogen and fibrin are anti-adhesive for keratinocytes: a mechanism for fibrin eschar slough during wound repair. J Invest Dermatol 2001;117(6):1369–81, an Elsevier publication, with permission.*

collagenase matrix metalloproteinase 1 (MMP-1), as well as plasminogen and, therefore, facilitates the degradation of interstitial collagen as well as provisional matrix proteins. Interestingly, keratinocytes in direct contact with collagen greatly increase the amount of MMP-1 they produce compared to that produced when they reside on laminin-rich basement membrane or purified laminin [28]. The migrating epidermis of superficial skin ulcers and burn wounds, in fact, express high levels of MMP-1 mRNA in areas where it presumably comes in direct contact with dermal collagen [29]. A more comprehensive review of ECM attributes, as well as their deposition and degradation, during reepithelialization has been published recently [24].

One to two days after injury, epithelial cells at the wound margin begin to proliferate. Although the exact mechanism is still not clear, both proliferation and migration of epithelial cells may be triggered by the absence of neighboring cells at the wound margin (the "free-edge effect"). The "free-edge effect" in the wound epidermis may be secondary to the modulation of cadherins junctions as described for V-cadherins during angiogenesis [30]. In fact, studies indicate that epidermal desmosomes lose their hyperadhesiveness and cadherins switch from E-cadherins to P-cadherins at the wound edge [31]. Other possibilities, not exclusive of the former, are growth factors that induce epidermal migration and proliferation and/or increased expression of growth factor receptors. These growth factors find their way to epidermal cells by autocrine, juxtacrine, hormonal, or paracrine pathways. For example, transforming growth factor (TGF)-α and -β, which originate from keratinocytes themselves, can act either directly on the producer cell or on adjacent epidermal cells in an autocrine or juxtacrine manner, respectively. By contrast, insulin-like growth factor comes from the circulation and thereby act as a hormone, while other growth factors, such as heparin-binding epidermal growth factor (HB-EGF) and keratinocyte growth factor, are secreted from macrophages and dermal parenchymal cells, respectively, and act on epidermal cells through paracrine pathways of epidermal/dermal bidirectional networks [32]. As evidence of their importance, many of these growth factors have been shown to stimulate reepithelialization in animal models [5]. Furthermore, lack of some of these growth factors or their receptors in knockout mice support the hypothesis that their activation of keratinocytes is required for optimal epidermal migration and/or proliferation during normal wound healing [33]. It appears that growth factor stimulation of JNK signal transduction pathways may be the key for "resetting" the epidermal program from differentiation to proliferation, and possibly migration [34].

As reepithelialization progresses, basement membrane proteins reappear in an ordered sequence from the margin of the wound inward in a zipper-like fashion [5]. Epidermal cells revert to their normal phenotype once again firmly attaching to reestablished basement membrane through hemidesmosomal proteins, including $\alpha 6\beta 4$ integrin, laminin 332 (aka laminin 5), and 180 kDa bullous pemphigoid antigen [35]. At this stage, they reattach to the underlying neodermis through type VII collagen fibrils [36]. A more detailed review of cutaneous wound reepithelialization has been recently published [37].

Granulation tissue

New stroma, often called granulation tissue, begins to form approximately 4 days after injury. The name derives from the granular appearance of newly forming tissue when it is incised and visually examined. It is numerous new capillaries (neovasculature) that endow the neostroma with its granular appearance. Movement of macrophages, fibroblasts, and blood vessels into the wound space as a unit underscores the biologic interdependence of these cells during tissue repair. Macrophages provide a continuing source of cytokines necessary to stimulate fibroplasia and angiogenesis, fibroblasts construct new ECM necessary to support cell ingrowth, and blood vessels carry oxygen and nutrients necessary to sustain cell metabolism [38]. The quantity and quality of granulation tissue depends on biologic modifiers present, the activity

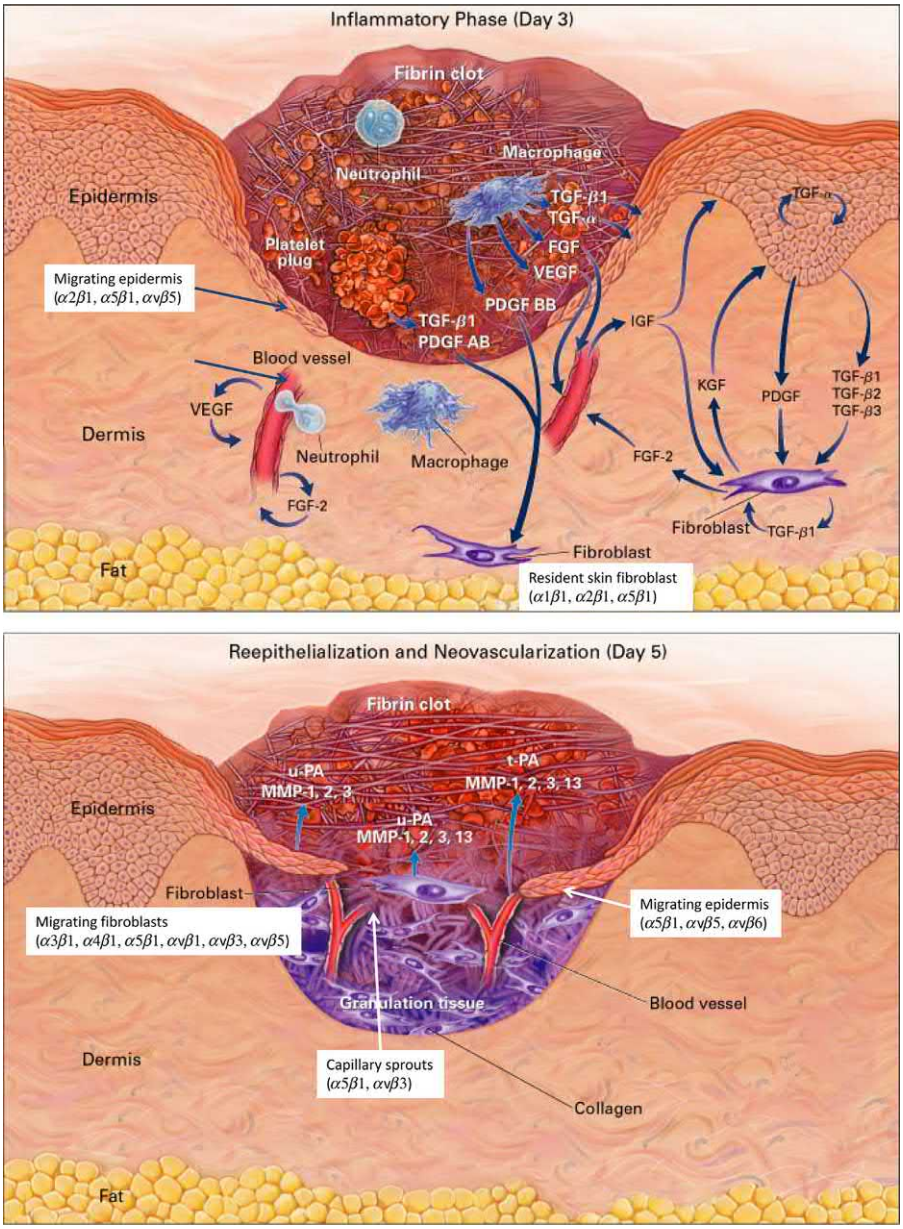

FIGURE 70.2 After injury the platelets, inflammatory cells, and the tissue cells (namely, fibroblasts, endothelial, and epidermal cells) secrete abundant quantities of multiple growth factors thought to be necessary for cell movement into the wound. However, it is the provisional matrix integrin expression that acts as the rate-limiting step in granulation tissue induction. Once the appropriate integrins are expressed on periwound endothelial cells and fibroblasts on day 3, the cells invade the wound space shortly thereafter (on days 4 and 5). Fibroblasts and endothelial cells express the fibrinogen/fibrin receptor avb3 and therefore are able to invade the fibrin clot; the epidermis, however, does not express avb3 and therefore dissects under the clot. Proteinases play an important role during granulation tissue formation by clearing the path for migrating tissue cells. Ultimately, the clot that has not been transformed into granulation tissue by invading fibroblasts and endothelial cells is dissected free of the wound and sloughed as eschar. *Modified from Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med 1999;341 (10):738–46 with permission.*

level of target cells, and the ECM environment. As mentioned in the "Inflammation" section, the arrival of peripheral blood monocytes and their activation into different subtypes of macrophages (see the "Transition from Inflammation to Repair" section) establish conditions for continual synthesis and release of growth factors. In addition, and perhaps more importantly, injured and activated parenchymal cells, for example, fibroblasts and epidermal cells, can synthesize and secrete growth factors. For example, migrating wound epidermal cells produce vascular endothelial cell growth factor (VEGF), TGF-β and PDGF-BB, to which endothelial cells and fibroblasts respond, respectively. The provisional ECM also promotes granulation tissue formation by positive feedback regulation of integrin ECM receptor expression [39]. Once fibroblasts and endothelial cells express the appropriate integrin receptors, they invade the fibrin/fibronectin-rich wound space (Fig. 70.2). Although it has been recognized for many years that ECM modulates cell differentiation by signal transduction from ligation of ECM receptors, more recently it has become evident that the force and geometry of the ECM influence cell behavior and differentiation [40–42].

Fibroplasia

Components of granulation tissue derived from fibroblasts including the cells themselves and the ECM are

collectively known as fibroplasia. Growth factors, especially PDGF and TGF-β, in concert with fibronectin as well as other provisional matrix molecules [43] presumably stimulate fibroblasts of the periwound tissue to proliferate, express appropriate integrin receptors, and migrate into the wound space. Many of these growth factors are released from macrophages or other tissue cells [5,6]; however, fibroblasts themselves can produce growth factors to which they respond in an autocrine fashion [44]. Multiple complex interactive biologic phenomena occur within fibroblasts as they respond to wound cytokines, including the induction of additional cytokines and modulation of cytokine receptor number or affinity. *In vivo* studies support the hypothesis that growth factors are active in wound repair fibroplasia. Several studies have demonstrated that PDGF, connective tissue factor, TGF-α, TGF-β, HB-EGF, and fibroblast growth factor (FGF) family members are present at sites of tissue repair [45–48]. Furthermore, purified and recombinant-derived growth factors have been shown to stimulate wound granulation tissue in normal and compromised animals [5], and a single growth factor may work both directly, and indirectly, by inducing the production of other growth factors *in situ* [49].

Structural molecules of the early ECM coined provisional matrix [50], contribute to tissue formation by providing a scaffold or conduit for cell migration (fibronectin) [51], low impedance for cell mobility (hyaluronan) [52], a reservoir for growth factors and cytokines [43,53], and direct signals to the cells through integrin receptors [25]. Fibronectin appearance in the periwound environment as well as the expression of fibronectin receptors appear to be critical rate limiting steps in granulation tissue formation [54]. In addition, a dynamic reciprocity between fibroblasts and their surrounding ECM creates further complexity [55]. That is, fibroblasts affect the ECM through new synthesis, deposition, and remodeling of the ECM while the ECM affects fibroblasts by regulating their function including their ability to synthesize, deposit, remodel, and generally interact with the ECM [39,56]. Thus the reciprocal interactions between ECM and fibroblasts dynamically evolve during granulation tissue development.

As fibroblasts migrate into the wound space, they initially penetrate the blood clot composed of fibrin and lesser amounts of fibronectin and vitronectin. Fibroblasts presumably require fibronectin in vivo for movement from the periwound collagenous matrix into the fibrin/fibronectin-laden wound space as they do in vitro for migration from a three-dimensional collagen gel into a fibrin gel [51]. Fibroblasts bind to fibronectin through receptors of the integrin superfamily (Table 70.1 *original*). The Arg–Gly–Asp–Ser (RGDS) tetrapeptide within the cell-binding domain of these proteins is critical for binding to the integrin receptors α3β1, α5β1, αvβ1, αvβ3, and αvβ5. In addition, the CSIII domain of fibronectin provides a second binding site for human dermal fibroblasts via the α4β1 integrin receptor [57]. In vivo studies have shown that arginine–glycine–asparatic acid (RGD)-dependent fibronectin receptors, α5β1 and αvβ3, are upregulated on periwound fibroblasts the day prior to granulation tissue formation and on early granulation tissue fibroblasts as they infiltrate the provisional matrix-laden wound [39]. In contrast the non-RGD-binding α1β1 and α2β1 collagen receptors on these fibroblasts were either suppressed or did not appear to change appreciably [39,58]. Furthermore, PDGF increases α5β1and α3β1 while decreasing α1β1 in cultured human dermal fibroblasts surrounded by fibronectin [58]. In addition, fibronectin or fibrin-rich environments promote the ability of PDGF to increase α5β1 and α3β1, but not α2β1 by increasing mRNA stability and steady-state levels [39]. These data strongly suggest that the type of integrin increased by PDGF stimulation appears to depend on the ECM context and suggests a positive feedback between ECM and ECM receptors.

Movement into a cross-linked fibrin blood clot or any tightly woven ECM may also necessitate active proteolysis to cleave a path for migration. A variety of fibroblast-derived enzymes in conjunction with serum-derived plasmin are potential candidates for this task, including plasminogen activator, interstitial collagenase-1 and -3 (MMP-1 and MMP-13, respectively), the 72 kDa gelatinase A (MMP-2), and stromelysin (MMP-3). In fact, high levels of immunoreactive MMP-1 has been localized to fibroblasts at the interface of granulation tissue with eschar in burn wounds [29] and many stromal cells stain for MMP-1 and MMP-13 in chronic ulcers [59]. TGF-β downregulates this proteinase activity, while PDGF stimulates the production and secretion of these proteinases [60]. From elegant knockout mouse studies, it is clear that the plasminogen activating system is critical for clearing the wound clot [61]. However, double knockout of MMP-13 (also called collagenase-3) and the plasminogen activating system in mice created more delay of healing compared to knockout of only the plasminogen activating system [62]. Furthermore, galardin, a broad-spectrum MMP inhibitor, slowed cutaneous healing, while MMP-9 deficient mice demonstrate altered fracture repair [63] and a knockout of MMP-8 (also called collagenase-2) adversely effected cutaneous wound repair [64]. Thus although there is great overlap in MMP function in tissue repair, their activity is clearly necessary for proper healing of cutaneous wounds.

When fibroblasts have completed their migration into the wound site, they switch their major function to protein synthesis [65]. Hence, the migratory phenotype is completely supplanted by a profibrotic phenotype characterized by

decreased α3β1 and α5β1 provisional matrix receptor expression, increased α2β1 collagen receptor expression, and abundant rough endoplasmic reticulum and Golgi apparatus filled with new collagen protein [39,65]. Accordingly, the fibronectin-rich provisional matrix is gradually supplanted with a collagenous matrix [56,65]. Under these conditions, PDGF, which is still abundant in these wounds [66], stimulates extremely high levels of α2β1 collagen receptor, but not α3β1 or α5β1 provisional matrix receptors, supporting the contention that the ECM provides a positive feedback for integrin expression [39]. TGF-β observed in wound fibroblasts at this time [56] can induce fibroblasts to produce great quantities of collagen [67]. IL-4 also can induce a modest increase in types I and III collagen production [68]. Since IL-4 producing mast cells are present in healing wounds, as well as fibrotic tissue, these cells may contribute to collagen matrix accumulation at these sites. Not surprisingly, the effect of IL-4 on healing tissue is strikingly dose and time dependent [69], as are the biological effects of most other cytokines and growth factors.

Mechanical stimuli have also been well-documented to induce fibroblast proliferation, migration, as well as production of growth factors, such as FGF, and TGF-β, and ECM proteins such as collagen [70,71]. These results have been shown both in vitro by subjecting fibroblasts to cyclical stretch and in vivo where negative pressure therapy stretches cells and can promote healing. Cells transduce these mechanical cues through integrins, ion channels, and growth factor receptors, which activate or modulate different signaling pathways. Integrins, in part, transduce their signals through focal adhesion kinase (FAK). Interestingly, knockout mice targeting fibroblast FAK experienced less inflammatory cell migration and fibrosis [70]. This evidence suggests mechanotransduction of cells, especially fibroblasts, during healing can modify fibroplasia to the benefit or detriment of healing, likely secondary to the degree and frequency of strain across the cells.

Once an abundant collagen matrix is deposited in the wound, fibroblasts cease collagen production despite the continuing presence of TGF-β [56]. The stimuli responsible for fibroblast proliferation and matrix synthesis during wound repair were originally extrapolated from many in vitro investigations and then confirmed by in vivo manipulation of wounds [20]. Both in vitro and *in vivo* studies suggest that gamma-interferon may be one such factor [72]. In addition, collagen matrix can suppress both fibroblast proliferation and fibroblast collagen synthesis [56,73]. In contrast a fibrin or fibronectin matrix has little or no suppressive effect on the mitogenic or synthetic potential of fibroblasts [56,74].

Although the attenuated fibroblast activity in collagen gels is not associated with cell death, many fibroblasts in day 10 healing wounds develop pyknotic nuclei [75], a cytological marker for programmed cell death (apoptosis). These results suggest that apoptosis is the mechanism responsible for the transition from a fibroblast-rich granulation tissue to a relatively acellular scar. Signal(s) causing apoptosis in wound fibroblasts have not been delineated, possibly attributable to the fact that in vitro experimental environments have not recapitulated the maturing wound environment, which may be required for priming wound fibroblasts for apoptosis [76]. Interestingly, fibroblasts in fibrotic diseases, such as keloid formation, morphea, and scleroderma, are also resistant to apoptotic signals and these signals may be disrupted either directly or indirectly [77].

Neovascularization

Fibroplasia would cease if neovascularization failed to accompany the newly forming complex of fibroblasts and ECM. The process of new blood vessel formation is called angiogenesis [78]. Many soluble factors that stimulate angiogenesis in wound repair have been elucidated [79]. Angiogenic activity can be recovered from activated macrophages as well as epidermal cells, fibroblast, endothelial cells, and numerous tumor cells [80]. Most biologically important angiogenic molecules have been identified and include vascular endothelial growth factor (VEGF), FGF-1 and FGF-2, TGF-α, TGF-β, TNF-α, platelet factor-4 (PF-4), angiogenin, angiotropin, angiopoietin, IL-8, PDGF, and low molecular weight substances including bioactive peptides, low oxygen tension, biogenic amines, lactic acid, and nitric oxide (NO) [5,6]. Some of these factors, however, are intermediaries in a single angiogenesis pathway, for example, TNF-α induces PF-4 that stimulated angiogenesis through NO [81]. Even more important, low oxygen tension stabilizes hypoxia inducible factor-1α (HIF-1α) that induces increased expression of VEGF [82]. To further complicate matters, not all growth factors within a family stimulate angiogenesis equally. For example, of four VEGF isoforms (VEGF-A, -B, -C, and -D) and 3 receptors (VEGFR1/Flt-1, VEGFR2/KDR/Flk-1, and VEGFR3), VEGF-A does not interact with VEGFR1 and VEGFR2 equally, and the signal transduction stimulated is not the same [83]. Furthermore, VEGF-C and -D stimulate lymphangiogenesis, rather than angiogenesis, through VEGFR3. Another complexity is that different growth factors effect blood vessel development at different stages. For example, VEGF-A stimulates nascent sprout angiogenesis, while angiopoietin induces blood vessel maturation [84].

Angiogenesis cannot be directly related to proliferation of cultured endothelial cells since endothelial cell migration is also required. In fact, Folkman and Shing [85] postulated that endothelial cell migration can induce proliferation. If true, endothelial cell chemotactic factors

may be critical for angiogenesis. Some factors, however, have both proliferative (mitogenic) and chemotactic (motogenic) activities, for example, PDGF [86] and EGF [87] are both motogenic and mitogenic for dermal fibroblasts, while VEGF is both motogenic and mitogenic for endothelial cells [88].

Besides growth factors and chemotactic factors an appropriate ECM is also necessary for angiogenesis. Three-dimensional ECM protein gels provide a more natural environment for cultured endothelial cells than monolayer protein coats [79] as is true for many other cultured cells [89]. Not surprisingly, different ECM proteins induce differential cell responses. For example, rat epididymal microvascular cells cultured in type I collagen gels with TGF-β produce capillary-like structures within 1 week [78]. Omission of TGF-β markedly reduces the effect. In contrast, laminin-containing gels in the absence of growth factors induce human umbilical vein and dermal microvascular cells to produce capillary-like structures within 24 hours of plating [90]. Matrix bound thrombospondin also promotes angiogenesis [91] possibly through its ability to activate TGF-β [92]. Although type I collagen does not induce angiogenesis without other contributing factors, it can protect newly formed blood vessels from apoptotic effects of angiostatic agents [93]. Together, these studies support the hypothesis that the ECM plays an important role in angiogenesis. Consonant with this hypothesis, angiogenesis in the chick chorioallantoic membrane is dependent on the expression of αvβ3, an integrin that recognizes fibrin and fibronectin, as well as vitronectin [94]. Furthermore, in porcine cutaneous wounds, αvβ3 is only expressed on capillary sprouts as they invade the fibrin clot [95]. In vitro studies demonstrate that αvβ3 can promote endothelial cell migration on provisional matrix proteins [96] and that human dermal microvascular endothelial cells can generate capillary sprouts in fibrin gels (αvβ3-dependent adhesion and migration) but not in collagen gels (α1β1- and α2β1-dependent adhesion and migration) [97].

Given the information outlined above, a series of events leading to angiogenesis can be hypothesized. Substantial injury causes tissue-cell destruction and hypoxia. Potent angiogenesis factors such as FGF-1 and FGF-2 are released secondary to cell disruption [98] while VEGF is induced by hypoxia. Proteolytic enzymes released into the connective tissue degrade ECM proteins. Specific fragments from collagen, fibronectin, and elastin, as well as many phylogistic agents, recruit peripheral blood monocytes to the injured site where these cells become activated macrophages that release more angiogenesis factors. Certain angiogenic factors, such as FGF-2, stimulate endothelial cells to release plasminogen activator and procollagenase. Plasminogen activator converts plasminogen to plasmin and procollagenase actives collagenase. These two proteases in concert digest basement membrane constituents.

The fragmentation of the basement membrane allows endothelial cells to migrate into the injured site in response to FGF, fibronectin fragments, heparin released from disrupted mast cells and other endothelial cell chemoattractants. To migrate into the fibrin/fibronectin-rich wound, endothelial cells express αvβ3 [94] and αvβ5 integrin [99]. Newly forming blood vessels first deposit a provisional matrix containing fibronectin and proteoglycans but ultimately form basement membrane. TGF-β may induce endothelial cells to produce the fibronectin and proteoglycan provisional matrix as well as assume the correct phenotype for capillary tube formation. FGF, and other mitogens such as VEGF, stimulate endothelial cell proliferation, resulting in a continual supply of endothelial cells for capillary extension. Capillary sprouts eventually branch at their tips and join to form capillary loops through which blood flow begins. New sprouts then extend from these loops to form a capillary plexus. Angiopoietin [100] and its recruitment of pericytes [101] are together important for the maturation and stabilization of newly formed capillaries. Furthermore, pericytes provide an important stem cell niche or act as stem cells themselves [102].

Within a day or two after the removal of angiogenic stimuli, capillaries undergo regression as characterized by mitochondria swelling in the endothelial cells at the distal tips of the capillaries, platelet adherence to degenerating endothelial cells, vascular stasis, endothelial cell necrosis, and ingestion of the effete capillaries by macrophages. Although αvβ3 can regulate apoptosis of endothelial cells in culture and in tumors [94], αvβ3 is not present on wound endothelial cells as they undergo programmed cell death indicating that their absence may be critical or that other integrins are involved. It is fairly clear that thrombospondin [103] or other ECM molecules are good candidate ligands for controlling endothelial cell apoptosis [104].

Wound contraction and extracellular matrix organization

During the second and third week of healing, fibroblasts begin to assume a myofibroblast phenotype characterized by large bundles of actin-containing microfilaments along the cytoplasmic face of the plasma membrane and the establishment of cell−cell and cell−matrix linkages [65,105]. In some [75], but not all [65], wound situations myofibroblasts express smooth muscle actin. Importantly, TGF-β can induce cultured human fibroblasts to express smooth muscle actin and may also be responsible for its expression in vivo [106].

The appearance of the myofibroblasts corresponds to the commencement of connective tissue compaction and the contraction of the wound. Fibroblasts link to the extracellular fibronectin matrix through α5β1 [65]; to collagen matrix through α1β1 and α2β1 collagen receptors [107]; and to each other through direct adherens junctions [65]. Fibroblast α2β1 receptors are markedly upregulated in 7-day wounds [39], a time when new collagenous matrix is accumulating and fibroblasts are beginning to align with collagenous fibrils through cell−matrix connections [65]. New collagen bundles in turn have the capacity to join end-to-end with collagen bundles at the wound edge and to ultimately form covalent cross-links among themselves and with the collagen bundles of the adjacent dermis [108]. These cell−cell, cell−matrix, and matrix−matrix links provide a network across the wound whereby the traction of myofibroblasts on their pericellular matrix can be transmitted across the wound [109].

Cultured fibroblasts dispersed within a hydrated collagen gel provide a reasonable functional in vitro model of wound contraction [110]. When serum is added to the admixture, contraction of the collagen matrix occurs over the course of a few days. When observed with time-lapse microphotography, collagen condensation appears to result from a "collection of collagen bundles" executed by fibroblasts as they extend and retract pseudopodia attached to collagen fibers [111]. More recent elegant studies have further defined fibroblast−collagen interactions [112]. The transmission of these traction forces across the in vitro collagen matrix depends on two linkage events: fibroblast attachment to the collagen matrix through the α2β1 integrin receptors [113] and cross-links between the individual collagen bundles [114]. This linkage system probably plays a significant role in the in vivo situation of wound contraction as well. In addition, cell−cell adhesions appear to provide an additional means by which the traction forces of the myofibroblast may be transmitted across the wound matrix [105]. Furthermore, gap junctions between wound fibroblasts probably provide the mechanism for contraction control across the cell population [115].

F-actin bundle arrays, cell−cell and cell−matrix linkages, and collagen cross-links are all facets of the biomechanics of ECM contraction. The contraction process, however, needs a cytokine signal. For example, cultured fibroblasts mixed in a collagen gel contract the collagen matrix in the presence of serum, PDGF, or TGF-β. Since TGF-β factor, but not PDGF, persists in dermal wounds during the time of tissue contraction, it is the most likely candidate for the stimulus of contraction [5]. Nevertheless, it is possible that both PDGF and TGF-β signal wound contraction: one more example of the many redundancies observed in the critical processes of wound healing. In summary, wound contraction represents a complex and masterfully orchestrated interaction of cells, ECM, and cytokines.

Collagen remodeling during the transition from granulation tissue to scar is dependent on continued collagen synthesis and collagen catabolism. The degradation of wound collagen is controlled by a variety of collagenase enzymes from macrophages, epidermal cells, and fibroblasts. These collagenases are specific for particular types of collagens, but most cells probably contain two or more different types of these enzymes [116]. Three MMPs have been described that have the ability to cleave native collagen: MMP-1 or classic interstitial collagenase that cleaves types I, II, III, X, and XIII collagens; neutrophil collagenase (MMP-8); and a novel collagenase produced by breast carcinomas that is prominent in chronic wounds (MMP-13) [59]. Currently it is not clear which *interstitial* collagenases are critical in the remodeling stage of human wound repair. For example, no wound-healing defect was observed in mice deficient of MMP-13 [117]; however, a double knockout of MMP-13 and the plasminogen activating system created an additional delay of healing compared to knockout of only the plasminogen activating system [62]. These findings are likely attributable to the redundancy of nature.

Cytokines such as TGF-β, PDGF, and IL-1, and the ECM itself clearly play an important role in the modulation of collagenase and tissue inhibitor of metalloproteinase (TIMP) expression in vivo. Interestingly, type 1 collagen induces MMP-1 expression through the α2β1 collagen receptor while suppressing collagen synthesis through the α1β1 collagen receptor [118]. Type I collagen also induces expression of α2β1 receptors [39], thus collagen can induce the receptor that signals a collagen degradation−remodeling phenotype. Such dynamic, reciprocal cell−matrix interactions appears to occur generally during tissue formation and remodeling processes such as morphogenesis, tumor growth, and wound healing to name a few [89].

Wounds gain only about 20% of their final strength by the third week, during which time fibrillar collagen has accumulated relatively rapidly and has been remodeled by myofibroblast contraction of the wound. Thereafter, the rate at which wounds gain tensile strength is slow, reflecting a much slower rate of collagen accumulation. In fact, the gradual gain in tensile strength has less to do with new collagen deposition than further collagen remodeling with the formation of larger collagen bundles and an accumulation of intermolecular cross-links. Nevertheless, wounds fail to attain the same breaking strength as uninjured skin. At maximum strength a scar is only 70% as strong as intact skin.

Chronic wounds

Acute wounds are those that heal through the routine processes of inflammation, tissue formation and remodeling,

which occur in a timely fashion. As discussed earlier, these processes may overlap temporally. However, prolonged continuance of any of these reparative processes may result in the formation of a chronic wound. Chronic wounds are often associated with underlying pathological conditions that contribute to an impaired healing. Venous leg ulcers and diabetic foot ulcers are common examples of chronic wounds caused or accentuated by an underlying disorder. While the former is being induced by insufficient venous flow that results in increased blood pressure in the lower limb microvascular bed and, therefore, increased vascular permeability, the latter is caused by peripheral neuropathy that leads to abnormal load distribution on the foot surface and decreased sensation [119]. Subsequently, these abnormalities cause a loss of tissue viability, suboptimal local tissue permeability, and an elevated and sustained inflammatory response.

Scarring

Injuries that require more time to healing, that is, an extensive or a chronic wound secondary to infection or other underlying adverse tissue condition, will create greater scarring. Furthermore, the injury must be deep enough for scarring to occur. For example, human dermal injuries must reach at least 0.51 mm from the epidermis for a noticeable scar to be observed 28 weeks later [120]. Scars can become problematic cosmetic or functional impediments depending on the location. For instance, a facial scar can cause psychological distress due to its appearance and functional impediment restricting facial muscle activity [17].

Scars are visibly distinct compared to normal skin due to their difference in architecture. Healthy skin is composed of chiefly type I collagen arranged in a "basketweave" pattern, whereas scar tissue is composed of type I collagen disposed in densely packed, parallel bundles secondary to wound contraction [14]. The compacted, parallel array of collagen bundles in scars compared to the "basketweave" architecture of collagen fibers in normal dermis, in part, explains why scars have about 20%−70% tensile strength compared to healthy tissue at 3 weeks and 6 weeks postinjury, respectively [70]. The difference in collagen bundle architecture also partly explains the difference in elasticity between normal skin and scar, as the basketweave pattern in healthy tissue allows for moderate stretch [121]. In addition, the lack of elasticity in scar is also secondary to the almost total lack of elastin in scars compared to normal skin.

Scars are also visibly different than normal skin due to the loss of both epidermal and dermal structures after injury. Severe skin injury, especially full-thickness damage as often occurs in burns, results in loss of melanocytes, absent or abnormal rete ridges in the epidermis, and destruction of dermal structures such as hair follicles, sebaceous glands, eccrine glands, nerves, and normal blood vessel plexi. These dermal structures are not restored without specialized cells so instead collagen and other ECM proteins fills this space.

Pathological scars

Pathological scars have unusual characteristics and compositions compared to normal scars. The most common pathological scars are hypertrophic scars and keloids. Hypertrophic scars are clinically defined as scars that are raised but remain within the wound bed, while keloids are clinically defined as scars that are raised and grow outside the wound bed. These are not tumors but primarily inflammatory disorders of the wound repair process. Hypertrophic scars appear within 4 weeks, while keloids appear after 3 months after injury. Also, after surgical intervention, hypertrophic scars tend not to recur, whereas keloids are known to recur [122]. These types of scars have become more common as more patients survive massive injury that in the past would be fatal [122]. A field that has seen a decrease in patient mortality but is accompanied with a rise in pathological scarring is in burn patients. Patients who have sustained high total body surface area burns are much more likely to survive but 70% of survivors develop severe pathological scars [123]. Beyond burn, patients can develop pathological scars after surgical procedures, but this is uncommon relative to postoperative normal scars [17].

It has been suggested that pathological scars develop due to chronic inflammation in the reticular dermis, mainly due to the observation that wounds that do not reach the reticular dermis do not form hypertrophic scars or keloids [124]. Local and genetic factors that augment inflammation are thought to influence hypertrophic scar and keloid development. Local factors such as rewounding and infection can result in a prolonged inflammation period, but the most significant local factors for pathological scar development are mechanical forces. Keloids are known to develop into predictable shapes depending on scar location and only develop on certain parts of the body. Modeling studies provide evidence that keloids adopt these shapes parallel to the direction of tension in the skin and only on skin that is frequently mechanically loaded [124]. Cells sense mechanical cues in the ECM such as tension, compression, or shear forces. Mechanotransduction of these cues signal the cell to respond in a variety of ways, such as proliferation, migration, and elongation, contributing to excessive fibrosis [71]. These mechanical signals also may contribute to a sustained myofibroblast phenotype that results in excessive contracture as observed in hypertrophic scars [70].

TABLE 70.2 Cellular and extracellular matrix (ECM) characteristics of normal scars, pathological scars, and scarless regeneration.

		Normal scarring	Hypertrophic scars	Keloids	Scarless regeneration
Cells	Myofibroblasts	Apoptose	Abundant and prolonged presence	Persistent	Absent
	Inflammatory cells and cytokines	↑	↑↑	↑↑	↓
ECM	TGF-β	TGF-β1	TGF-β1, TGF-β2	TGF-β1, TGF-β2	TGF-β3
	Collagen type	Type I > type III	↑ Type III and type V	↑ Type I	Type III > type I
	Collagen orientation	Parallel to epidermis	Parallel to epidermis	Disorganized	Basketweave
	Collagen bundle thickness	Small, parallel bundles	Thin nodules	Thick nodules	Small, thin bundles
	Collagen cross-linking	↓	↑	↑↑	↓
	Elastin	↓	↓	↑↑	↑
	Mechanical strain	↑	↑↑	↑	↓

TGF, Transforming growth factor.

Pathological scarring has thought to be determined by genetics as well. For example, many patients with pathological scars have a family history of pathological scarring, and scars are much more likely to develop in darker skinned and Asian individuals [70]. Notably, pathological scars never develop in albinos [124]. Unfortunately, not much research has been conducted to illuminate the genes that impact pathological scarring. However, the current prevailing theory is that single nucleotide polymorphisms are the cause for the differences in predisposition for pathological scarring [124]. In a recent study a specific variant of the CSMD1 gene that was predominately found in white males was linked to a lower likelihood of hypertrophic scarring [123].

An imbalance of MMPs may lead to an imbalanced remodeling process where too much collagen is deposited that cannot be properly remodeled. This results in a disproportionate ratio of collagen, contributing to pathological scars. Hypertrophic scars are reported to express less MMP-1, MMP-2, MMP-9, and MMP-13 than normal scars [121]. Similarly, keloids express low levels of these MMPs, save for MMP-9 which is found at comparable concentrations as normal scar [125]. In both hypertrophic scars and keloids, tissue inhibitor of matrix metalloproteases 1 and 2 (TIMP-1 and TIMP-2) are increased compared to normal scar. This leads to hypertrophic scars having 33% type III and 10% type V collagen organized parallel to the epidermis in thin nodules and keloids to have much more type I and little type III collagen disorganized in thicker nodules. In both cases, collagen synthesis is much higher than in normal scars. Fibroblasts in hypertrophic scars and keloids deposit collagen seven times and twenty times faster than those in normal scars, respectively [125]. Both hypertrophic scars and keloids have higher concentrations of TGF-β receptor, TGF-β1, and TGF-β2 compared to normal scar. Hypertrophic scars also have more fibronectin and hyaluronan in the ECM, assisting cell migration [123]. The reddened appearance characteristic of pathological scars is due to dysfunctional angiogenesis and microvessel invasion. Microvessels infiltrate normal scar but eventually recede at the remodeling stage, but in pathological scars, they persist and become hypervascular [17]. Major differences in pathological scars compared to normal scars are summarized and can be found in Table 70.2.

Scarless healing

Scarring is an expected consequence of the need for wound healing in humans to be rapid and efficient to stave off infection. The only exception of scar formation postinjury in humans is the fetus and the oral mucosa, both of which can regenerate tissue damage without scar formation [70,121,126]. In these rare cases of scarless healing, also known as regeneration, the healed tissue is virtually identical to uninjured tissue, possessing the dermal structures, proper collagen type ratios, and collagen organization seen in healthy skin [70]. For fetal healing

the transition from regenerative healing to normal healing is around 24 weeks of gestation. Before this time point the fetus can regenerate the damage sustained with no scar formation. After this point, scar is formed such as normal adult wound healing. Scarless healing in the fetus follows the typical wound healing steps but begins with a much less effective and shorter inflammatory stage. During this stage, less neutrophils and macrophages migrate to the wound, and cytokines are released in different concentrations. For example, IL-6 and IL-8 concentrations are lower, whereas IL-10 concentration is significantly increased compared to concentrations found in adult wound healing [70]. Not only are cytokine concentrations different but growth factors are as well. Important growth factors in adult normal wound healing such as PDGF, TGF-β1, and TGF-β2 are significantly reduced and instead TGF-β3 is much higher [70,121]. In addition, all MMPs synthesized in the wound are upregulated, and TIMPs are downregulated, promoting collagen degradation and remodeling earlier on than normal healing. Interestingly, fetal fibroblasts can simultaneously deposit collagen and proliferate, whereas adult fibroblasts can only efficiently perform one task at a time [126]. The collagen deposited in fetal healing is organized as thin bundles of primarily type III collagen along with some type I collagen, of which has a low resting mechanical stress [126].

In adult humans the oral mucosa can heal with insignificant scar as well. This tissue has some similarities to fetal tissue in which the environment is consistently hydrated, experiences little mechanical stress, and has a stunted inflammatory response. Studies have been conducted transplanting dermal fibroblasts and oral mucosa fibroblasts to damaged tissue of the others' origin [70]. The oral mucosa fibroblasts transplanted into a skin wound healed with less scar than dermal fibroblasts that were transplanted into an oral mucosa wound. This suggests that intrinsic factors of cells also play a role in scarless healing.

Tissue engineered therapy with skin cells

Initial attempts to speed up wound repair and improve the quality of healing in chronic or burn wounds involved the use of synthetic, composite synthetic, or biological dressings [127]. Although temporarily effective, these dressings did not offer any permanent treatment since eventually, an autograft had to be implanted to achieve complete healing. The advent of tissue-engineered constructs has, however, benefited the wound healing care [128]. These constructs could be classified into two main categories: cellular and acellular. In both cases the basic building blocks are a biomimetic and a scaffolding material. While the biomimetic functions to stimulate cell recruitment and the desired cellular functions, the scaffold typically provides a mechanical support for the cells. Scaffolds prepared from naturally occurring biopolymers tend to provide the correct biological stimuli to support cell function and tissue formation [129]. Nevertheless, whether the scaffold is natural or synthetic, the goal is to promote faster healing that results in the development of a new tissue that bears structural and functional resemblance to the uninjured, host tissue. A comprehensive list of current cell therapeutics, biologic dressings, and skin substitutes for acute and chronic skin wounds has been recently published [7].

Engineered epidermal constructs

Immediate wound coverage, whether permanent or temporary, is one of the cornerstones of wound management. Doing so reduces the chances of infection, promotes wound closure, reduces chance of hypertrophic scarring, and reduces scar depth. Engineered epidermal constructs with attributes similar to those of autologous epidermis have been used to facilitate repair of partial-thickness wounds where the major damage is to the epidermis.

Cultured autologous keratinocyte grafts were first used in humans by O'Conner et al. [130]. Subsequently, there has been extensive experience with cultured epidermal grafts for the treatment of burns as well as other acute and chronic wounds [131,132]. Epicel (Genzyme Tissue Repair) is an example of an engineered epidermal autograft. The potential advantage of this technique is the ability to provide autologous grafts capable of covering large areas with reasonable cosmetic results. Another significant advantage of autologous grafts is their ability to serve as permanent wound coverage, since the host does not reject them. However, a major disadvantage is the several week lag between host skin harvest and the availability of sufficient quantities of keratinocytes, the need for an invasive procedure to obtain autologous donor cells, and the large costs incurred. Furthermore, graft take is widely variable based on wound status, patient age, general host status, and operator experience.

Cultured keratinocyte allografts were developed to help overcome the need for biopsy and separate cultivation for each patient to produce autologous grafts and the long lag period between epidermal harvest and graft product. Cultured epidermal cells from both cadavers and unrelated adult donors have been used for the treatment of burns [133] and chronic leg ulcers [134]. Although allografts made from neonatal foreskin keratinocytes were more responsive to mitogens than adult (cadaver) cells and, therefore, initially preferred for cultured allografts, a later investigation revealed that such allografts were more immunogenic than the regular culture skin substitutes and thus potentially problematic [135].

To facilitate mass allograft production and wide availability, cryopreserved allografts have been developed and were fairly comparable to fresh allografts [136]. Despite these advances, the culture epidermal grafts have failed to produce satisfactory response. The primary reasons include the lack of mechanical strength and graft site susceptibility to wound contractures. As an alternative, keratinocyte delivery systems were developed where the cells were delivered to the injury site via a biodegradable scaffold. For example, laser skin, produced by Fidia Advanced Biopolymer, Italy, is used to deliver keratinocytes via a chemically modified hyaluronan membrane, which is perforated with micron-sized holes that allow cells to grow to confluence.

Another approach has been to isolate fresh keratinocytes from the patients and then spray them onto the wound site [137,138]. For example, RECELL is a system developed by AVITA Medical that harvests autologous cells from a patients' donor site to create an autologous cell suspension that can be sprayed onto a wound bed with no cell culture involved [139]. RECELL treated wounds at 4 weeks showed comparable wound closure and scarring to control split-thickness autografts but also claim to reduce scar pain and pruritus at 16 weeks [139]. This system is advantageous over the current gold standard because it significantly reduces the amount of donor tissue to be harvested for an autograft. RECELL is reported to treat 80 cm^2 of area per 1 cm^2 of collected tissue [139,140]. One study saw a donor tissue reduction of 97.5% [139] and 32% in another [140], both with comparable outcomes to split-thickness grafts. In addition, the suspension consists of autologous tissue that provides the necessary cells such as fibroblasts, keratinocytes, and melanocytes in physiological concentrations to support regeneration and reepithelialization of the wound. Nevertheless, The National Institute for Health and Care Excellence found that healing and scar appearance were not significantly improved compared to split-thickness grafts, but RECELL did reduce the need of subsequent grafts, indicating that this system has potential to reduce autograft cost [141].

Engineered dermal constructs

While the use of cultured keratinocytes to enhance wound healing has met with modest success, it lacks a dermal component that, if present, would provide greater mechanical stability and possibly prevent wound contraction. Allografts containing dermis, for example, pig skin or cadaver skin, have been used for many years as temporary coverage, but they tend to induce an inflammatory response [142]. However, such skin can be chemically treated to remove the antigenic cellular elements (AlloDerm, LifeCell Corporation, Woodlands, TX) and used alone or in combination with cultured autologous keratinocytes for closure of various chronic wounds and burns [143]. In spite of these modifications, allogenic grafts, when compared with autologous grafts, have been shown to promote lower percent reepithelialization and excessive wound contraction [144]. Furthermore, the overall therapeutic outcome of a skin graft depends also on the site from which the cells were isolated (during biopsy) [145].

Burke et al. [146] developed an acellular composite skin graft made of a collagen-based dermal lattice (containing bovine collagen and chondroitin-6-sulfate) with an outer silicone covering. After placement on the wound the acellular dermal component recruits the host dermal fibroblasts while simultaneously undergoing degradation. About 2–3 weeks later the silicone sheet is removed and covered with an autograft. This composite graft has been used successfully to treat burns [147] and has received FDA approval for this indication as well as for reconstructive surgery (Integra, Integra Life Sciences Corporation, Plainsboro, NJ) [148]. However, these constructs cannot be used in patients who are allergic to bovine products.

Another rendition of a dermal substitute is TransCyte (Dermagraft-TC) (Advanced BioHealing, La Jolla, CA). This product consists of an inner nylon mesh in which human fibroblasts are embedded together with an outer silicone layer to limit evaporation. The fibroblasts are lysed in the final product by freeze–thawing. Prior to that time, the fibroblasts had manufactured collagen, matrix proteins, and cytokines all of which promote wound healing by the host. TransCyte has been used successfully as a temporary wound coverage after excision of burn wounds [149] and has been approved by the FDA for this indication. Dermagraft is a modification of this product in which a biodegradable polyglactin mesh is used instead of a silicone layer and the fibroblast remain viable [150].

Engineered skin substitutes

Full thickness wounds involve the loss of both the epidermal and dermal layers of the skin. To treat such extensive wounds, Bell et al. first described a bilayered skin composite consisting of a collagen lattice with dermal fibroblasts that was covered with epidermal cells [151]. Modification of this composite consisting of type I bovine collagen and live allogeneic human skin fibroblasts and keratinocytes has been developed (Apligraf, Organogenesis, Canton, MA). It has been used successfully in surgical wounds, venous leg ulcers, and diabetic foot ulcers [152], despite the fact that it is not a permanent skin replacement [153]. In a large multicenter trial, this product resulted in accelerated healing of chronic

nonhealing venous stasis ulcers when compared to standard compressive therapy [154].

StrataGraft, a product developed by Mallinckrodt, is a biomimetic construct composed of human neonatal immortalized keratinocytes (NIKS), complete with dermis and stratified epidermis [155]. This product can be sutured, stapled, or otherwise secured in the wound bed and serves to reduce infection, promote early wound closure, and optimize healing properties. These purposes can be achieved without the risk associated with allografts, such as disease transmission, because the NIKS cell line has been well-characterized and proven safe, eliciting no immune response or adverse reaction [156]. Currently, the FDA designates StrataGraft as a Regenerative Medicine Advanced Therapy as it is a therapeutic tissue-engineering product that can be used to fulfill an unmet, life-threatening clinical need. This designation places StrataGraft on a priority pathway for approval, and the FDA is anticipated to conclude its review of StrataGraft in 2020 after phase III trials, which focus on efficacy and safety after 3 months post StrataGraft application. Phase I and II trials of StrataGraft analyzed immune response at 1 and 2 weeks, wound closure at 3 months, and adverse effects up to 12 months after StrataGraft application, but the longer term appearance and quality of healing as well as patient safety when using these spontaneously immortalized cells have not been as thoroughly investigated.

Skin autograft harvesting without scarring

Another strategy for skin replacement is to use microcolumns of autologous skin for autologous grafting [157]. These microcolumns are about 400−700 μm in diameter and contain all the elements that make up human skin. In addition, these elements are in their natural anatomical locations relative to one another. The microcolumns can restore many functional parts of the skin, such as neurons and sweat glands, when grafted into a wound in animal models. Such a wide range of regeneration has only been seen with full-thickness skin grafts; however, harvesting full thickness skin results in damage to the donor site. In contrast the donor site of skin microcolumns has been observed to undergo full regeneration without scarring. In its current iteration the microcolumns have been placed into the wound with random orientations rather than having the upper layers of skin on top. However, split-thickness skin grafts lead to wound reepithelialization even when placed onto the wound upside down, if the wound is kept hydrated. Therefore the authors suggest that while preserving the proper orientation of the microcolumns may improve their efficacy, it is not critical for wound healing. Nevertheless, their ongoing work is focused on solving this problem.

Tissue-engineered therapy with stem cells, bioactives, and biomaterials

Although the increased healing rates observed with the use of these engineered constructs show promise for the treatment of burn and/or chronic wounds, they have several intrinsic disadvantages that limit their use—(1) the epidermal grafts are very fragile and therefore difficult to handle, (2) it is difficult to quality control the large-scale production of any cell-populated matrix, and (3) while autografts require skin biopsy, allografts may experience early rejection. Moreover, these constructs in general are only about 25% efficient, implying that they must be applied on at least four patients before their effect can be seen. These limitations suggest that further improvements be made so that tissue-engineered constructs are not only more effective but also less complex.

Two approaches emerge from the foregoing discussions. The first tissue-engineering design utilizes cells that resist rejection and/or provide a more robust clinical outcome that makes their use cost-effective despite the absolute costs required to produce them. Alternatively, second-generation, acellular tissue-engineered products are being developed that more effectively recruit host tissue cells into the wound and then provide biological signals for the accumulated tissue cells to induce tissue regeneration rather than scar. To address the first approach, many groups are trying to isolate the "right" stem cells to stimulate wound repair or preferably tissue regeneration. For the skin, both epidermal stem cells [19,158] and bone marrow- or adipose-derived mesenchymal stem cells (MSCs) have been isolated by numerous methods and delivered by many different methods [159,160]. For example, adipose tissue removed via liposuction can be enzymatically digested to get a stromal vascular fraction. ASCs and primary adipocytes can then be separated from this solution via centrifugation. Although these cell populations tend to aggregate, ASCs can be isolated by tissue culture [161]. New and better methods for isolating and standardizing MSCs are greatly needed and several investigators are assiduously tackling this problem [162]. Better understanding of the niche where stem cells normally reside will help elucidate the microenvironment that the cells need to self-renew versus differentiate along the appropriate path [163,164].

For the acellular "smart" matrix tissue-engineering approach, clues learned from embryogenesis, morphogenesis, and wound repair should be implemented to engineer revolutionary constructs that facilitate and synchronize tissue repair. The transformative acellular product must be conductive to rapidly recruit the host tissue cells and inductive to stimulate the invading cells to proliferate, synthesize new ECM, and then differentiate appropriately to regenerate the lost tissue.

Various engineered skin constructs have used collagen as the preferred scaffolding material for cell seeding [165]. The huge popularity of collagen can be attributed to its abundance in skin, its interaction with cells, and its ability to markedly increase mechanical strength to the skin through forming cross-links with itself and other extracellular matrix molecules [166]. However, during wound repair, collagen appears only during the later stages after the invading and proliferating fibroblasts have filled the wound space. Therefore collagen may not be optimal for initial cell migration. Since fibronectin and hyaluronan are present during early stages of embryogenesis, morphogenesis, and wound healing [20], these biomaterials or their derivatives are favored to design "smart" matrix for cutaneous wound repair.

Hyaluronan is a nonsulfated glycosaminoglycan that is present in most human tissues. During wound repair, it serves multiple important functions, ranging from regulating inflammation to promoting fibroblast migration and proliferation [167]. Interestingly, hyaluronan has been implicated in the scarless or minimally scarred repair of fetal wounds, perhaps owing to its role in regulating the inflammatory response [168] and collagen deposition [169]. Furthermore, similar to synthetic polymers, hyaluronan can be chemically modified to obtain a variety of stable derivatives [170]. Therefore by offering the advantages of both natural and synthetic materials, hyaluronan promises to be a more suitable scaffolding material for acellular matrices as compared to collagen. Indeed, chemically modified hyaluronan scaffolds have been successfully used for various tissue-engineering applications, including wound repair [171].

Since fibroblast migration is the rate-limiting step in granulation tissue formation [54], a biomaterial ± biomimetic must support maximal fibroblast migration. Fibronectin is a favorable candidate since (1) it appears together with hyaluronan at times of cell migration during embryogenesis, morphogenesis, and wound repair [20,52]; (2) fibroblast migration on hyaluronan/fibronectin gels is far greater (approximately fourfold) when compared to that on fibrin/fibronectin gels (Greiling and Clark, unpublished observation), (3) fibronectin has been shown to be required for fibroblast transmigration from a collagen gel to a fibrin gel [51], and (4) fibronectin is absent in chronic wounds, where it is produced normally [172] but eliminated rapidly by the abundant proteases present in chronic wound fluid [173,174]. Although fibronectin appears to be an ideal biomimetic for use in hyaluronan scaffolds, its stability in the proteolytic environment of chronic wounds is a major concern.

Alternatively, the proteolytically stable RGD peptide sequence, the smallest cell recognition sequence in the 10th module of type III repeat of fibronectin, can be used to support key cell functions [175]. RGD has been widely used to promote cell attachment and spreading in various tissue-engineering applications in general [176] and wound-healing applications in particular [177]. This is perhaps because the RGD sequence is found in a variety of ECM molecules and, therefore, recognized by the transmembrane integrin receptors of multiple cell types, including dermal and epidermal tissue cells [178]. Our previous study has shown that hyaluronan hydrogels decorated with RGDS support NIH 3T3 fibroblast functions in vitro [179] and when seeded with 3T3 fibroblasts and implanted in nude mice, produce granulation-like tissue in 4 weeks. Therefore RGD-modified hyaluronan hydrogels appeared to possess great inductive properties. However, these hydrogels neither supported optimal human adult dermal fibroblast functions nor demonstrated conductive properties required of any acellular scaffold [179].

To impart our construct with both inductive and conductive properties, we selected, as the biomimetics, three FN functional domains, namely, $FNIII_{(8\text{-}11)}$, $FNIII_{(12\text{-}15)}$, and $FNIII_{(12\text{-}V\text{-}15)}$, that are necessary and sufficient for optimal dermal fibroblast migration in vitro [180]. Indeed, these hydrogels were successful in supporting dermal fibroblast functions in vitro and promoting wound repair in vivo [181]. Since these hydrogels can be formulated at room temperature and physiological pH, they are compatible with both cells and the incorporated biological molecules. In addition, their rapid gelation (<10 minutes) advocates their possible injectable use. However, manufacturing a tissue-engineered implant containing three recombinant proteins raises concerns related to cost, quality control, sterilization, and shelf-like of the product. To solve such issues, we are currently working to develop fibronectin-derived peptides that substitute for the three domains previously used [182].

With a similar objective, several other groups have also developed "intelligent" scaffolds for tissue repair [183]. These approaches commonly employ synthetic materials to build scaffolds since they allow great flexibility during formulation. To impart bioactivity, these scaffolds contain potent biomimetics that can be recognized by tissue cells. However, as discussed earlier, cell invasion during granulation tissue formation occurs concurrently with matrix degradation, which can be typically observed when using naturally derived materials. To elicit a similar response in synthetic materials, protease-sensitive sequences are incorporated within the scaffold that are cleaved upon contact with the cell-secreted proteolytic enzymes [184,185]. Therefore these scaffolds combine the advantages of both natural and synthetic biomaterials to facilitate wound repair.

Traditionally, the structural component of the tissue-engineered constructs has been viewed as providing only a passive mechanical structure. This design reflects our common understanding that cells primarily respond to biological signals. However, over the past decade it has

become increasing clear that mechanical forces alone can govern cell and tissue phenotype in ways similar to biological stimuli [186]. Further studies have revealed that cells use an active tactile sensing mechanism to feel and respond to substrate mechanics [40,41] and that stem cells use clues from this mechanical sensing to select pathways for differentiation [12]. The latter observation was not too surprising since it had already been demonstrated that dermal fibroblasts respond to substrate mechanics by regulating levels of gene transcription that eventually lead to differential ECM synthesis and their transformation into myofibroblasts [109]. Since these processes are critical during wound repair, effective tissue-engineering approaches for wound repair would require optimization of both biological and mechanical effectors.

The acellular tissue-engineered constructs discussed so far utilize scaffolding materials to provide mechanical support for tissue ingrowth and biomimetics to induce key cell functions. The primary goal of these novel approaches is to mimic the attributes of fibrin clot for parenchymal cell migration. However, a fibrin clot is not only composed of a fibrin/fibronectin scaffold and an array of clotting and fibrinolytic enzymes, but also a plethora of growth factors that had been released during platelet aggregation [187]. Growth factors play a crucial role in the overall healing response where they function to stimulate cell migration, proliferation, differentiation, and angiogenesis. Furthermore, growth factor deficiency often leads to impaired wound repair [188]. As a result, several groups have investigated the use of tissue-engineered constructs for local growth factor delivery, where the release of appropriate growth factors produced an increase in angiogenic activity [189,190].

It is interesting to note that despite the release of growth factors immediately after wounding, there remains a 3-day lag before granulation tissue is formed. This suggests that the growth factors may be retained and functional within the clot. This form of "solid-state" biochemistry may be unconventional but is backed by studies that demonstrate that numerous growth factors, which are bound to specific molecular domains of FN, retain or have accentuated bioactivity [191–194]. Interestingly, while some investigators have shown that increased angiogenesis driven by excess activity of VEGF or other angiogenic factors actually decrease the healing of skin wounds [195], we have found a peptide cP12 from fibronectin that enhances PDGF-BB activity in vitro and also promotes faster burn wound healing in vivo [182]. Therefore by incorporating the appropriate growth factor-binding sequences/domains, tissue-engineered construct can be used as a growth factor repository, causing an increase in local concentration that may, in some cases, accentuate cell functions.

In conclusion, wound healing is a dynamic and fine-tuned cellular response aimed at reinstating tissue homeostasis after an insult. Vigorous cellular activities observed during wound repair are similar to those occurring during embryogenesis and morphogenesis, which indicates the enormous complexity of this physiological reparative process. That may also explain why despite over two decades of intense research and development, we have still not identified an "ideal" therapy. However, novel tissue-engineering approaches are showing tremendous promise and aiming to push the limits of human expectations of wound therapy.

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Chapter 71

Bioengineered skin constructs

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Introduction

Cutaneous wounds normally heal by the formation of epithelialized scar tissue rather than regeneration of full-thickness skin. Consequently, strategies for the clinical management of wound healing have depended historically on providing a passive cover to the site of the wound while allowing the reparative mechanisms of wound healing, including reepithelialization, remodeling of granulation tissue, and formation of scar tissue, to occur, and therapy could do little more than facilitate these processes. However, advances in our understanding of wound healing, wound assessment, the concerted action of several growth factors, the role of the extracellular matrix (ECM) in regulating the healing process, and the demonstrated ability of bioengineered constructs to promote wound healing highlight the potential for intervening therapeutically in tissue repair by providing new epithelium, stimulating dermal repair, and reconstituting full-thickness skin.

Bioengineered skin substitutes can be classified as either cell-based constructs that actively stimulate wound healing or acellular constructs that provide a substrate or covering to facilitate wound healing. Cell-based constructs include, though not exclusively, autologous epidermal cell sheets (Epicel, Vericel Corp.), allogeneic dermal substrates (Dermagraft, Organogenesis, Canton, MA), and human skin equivalents (HSEs) composed of both dermal and epidermal components (Apligraf, Organogenesis Inc., Canton, MA; Orcel, Ortec International Inc.; StrataGraft, Stratatech, Madison, WI) and acellular products (Transcyte, Shire Regenerative Medicine, San Diego, CA; Integra Dermal Regeneration Template, Integra Life Sciences, Plainsboro, NJ; Biobrane, UDL, Rockford, IL). Another construct, comprising cultured keratinocytes and fibroblasts in bovine collagen, is the living cellular sheet, GINTUIT (Organogenesis Inc., Canton, MA), indicated for topical application to a surgically created vascular wound bed in the treatment of mucogingival conditions. A recent publication summarizes many of the worldwide products (Int J Mol Sci. 2017 Apr; 18(4): 789). A difficulty is that there is considerable change in the companies manufacturing these products. Therefore one must keep in mind this flexibility. Although not all of these products are still commercially available, they represented the first of their kind and are the result of basic research in the biology of skin and wound healing and in clinical experience with skin grafts, cultured keratinocyte grafts, acellular collagen matrices, cellular matrices, and cultured composite grafts [1–4].

Skin structure and function

Skin has several distinct functions. As the interface between the environment and the body, skin provides a protective barrier from microbes, toxins, ultraviolet radiation, and abrasion/ulceration, while also preventing water loss. In addition, skin enables the body's ability to sense heat and cold, pressure, vibration, and pain and regulates body temperature through perspiration and blood flow. The passive and active functions of skin are carried out by specialized cells and structures located in its two main layers: the epidermis and the dermis (Fig. 71.1). Complex functional relationships between these two anatomic structures of skin maintain skin's normal properties. Tissue engineering applications in skin depend upon an understanding of the structural components of skin, the spatial organization, and the functional relationships of skin's components.

The epidermis

As stated earlier, skin is a physical barrier between the body and the external environment. The outermost layer

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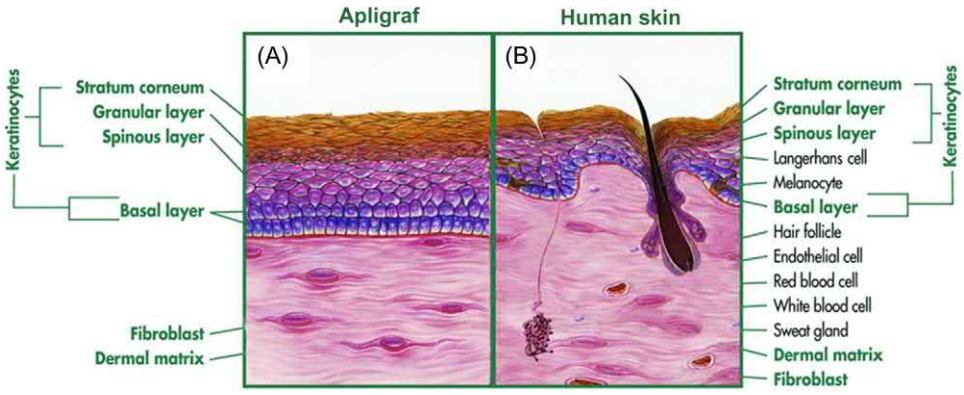

FIGURE 71.1 The basic components of skin and engineered skin equivalent.
(A) Diagram showing the major cell types of skin and their organization. Note that stratified keratinocytes make up the epidermis and display distinct morphological phenotypes. (B) A histological section of Apligraf HSE, (hematoxylin and eosin, 142×). Italics indicate cell types present in real skin but not in the engineered skin equivalent.

of skin, the epidermis, is the layer that is impermeable to toxic substances and harmful organisms. It is also the layer that controls the loss of water from the body to the relatively drier external environment.

The epidermis is composed primarily of keratinocytes, which form a stratified squamous epithelium (Fig. 71.1). Proliferating cells in the basal layer of the epidermis and structural components of the basements membrane anchor the epidermis to the dermis and replenish the terminally differentiated epithelial cells lost through normal sloughing from the surface of the skin. The basal cells stop proliferating and terminally differentiate into squamous keratinocytes as they move from the basal layer through the suprabasal layers to the surface of the epidermis. Keratin filaments and desmosomes contribute physical strength in the living layers and maintain the integrity of the epidermis. The cornified envelopes serve as the bricks and the lipids as mortar.

The most superficial keratinocytes in the epidermis form the stratum corneum, which represents the dead outermost structure that provides the physical barrier of the skin. In the last stages of differentiation, epithelial cells extrude lipids into the intercellular space to form the permeability barrier. The cells break down their nuclei and other organelles and form a highly cross-linked protein envelope immediately beneath their cell membranes. The physically and chemically resilient protein envelope connects to a dense network of intracellular keratin filaments to provide further physical strength to the epidermis.

Additional cells and structures in the epidermis perform specialized functions (Fig. 71.1). Skin plays a major role in alerting the immune system to potential environmental dangers. The interacting cells in skin comprise a dynamic network capable of sensing a variety of perturbations (trauma, ultraviolet radiation, toxic chemicals, and pathogenic organisms) in the cutaneous environment, and rapidly sending appropriate signals that alert and recruit other branches of the immune system [5,6]. To restore homeostasis in the skin immune system, the multiple proinflammatory signals generated by skin cells must eventually be counterbalanced by mechanisms capable of promoting resolution of a cutaneous inflammatory response. Dendritic cells of the immune system (Langerhans cells) reside in the epidermis and form a network of dendrites through which they interact with adjacent keratinocytes and nerves [7]. Melanocytes distribute melanin to keratinocytes in the form of melanosomes. Melanin protects the epidermis and underlying dermis from ultraviolet radiation. Sweat glands help to regulate body temperature through evaporation of sweat secreted onto the skin surface. Sebaceous glands associated with hair follicles secrete sebum, an oily substance that lubricates and moisturizes hair and epidermis. Hair keeps the body warm in many mammals, although maintaining body temperature is not an important role of hair in humans. Hair follicles, however, are an important source (a reservoir) of proliferating keratinocytes during reepithelialization after wounding.

The dermis

The dermis underlies the epidermis (Fig. 71.1). The dermis is divided into two regions: the papillary dermis, which lies immediately beneath the epidermis, and the deeper reticular dermis. The papillary dermis is composed of loose connective tissue and form papillae that intertwine with the rete ridges of the epidermis. The reticular dermis is more acellular and has a denser meshwork of thicker collagen and elastic fibers than the papillary dermis. The reticular dermis provides skin with most of its strength, flexibility, and elasticity. Loss of reticular dermis can often lead to excessive scarring and wound contraction.

The dermis provides physical strength and flexibility to skin as well as the connective tissue scaffolding that supports the extensive vasculature, lymphatic system, and nerve bundles. The dermis is relatively acellular, being composed predominantly of an ECM of interwoven collagen fibrils. Interspersed among the collagen fibrils are elastic fibers, proteoglycans, and glycoproteins.

Fibroblasts, the major cell type of the dermis, produce and maintain most of the ECM (Fig. 71.1). Fibroblasts in the papillary dermis and reticular dermis have greater proliferative and secretory activity, respectively. Endothelial cells line the blood vessels and play a critical role in the skin immune system by controlling the extravasation of leukocytes. Cells of hematopoietic origin in the dermis (e.g., macrophages and lymphocytes) contribute to a surveillance function. A network of nerve fibers extends throughout the dermis, which serves the sensory role in the skin (and, to a more limited extent, a motor function). These nerve fibers also secrete neuropeptides that influence immune and inflammatory responses in skin through their effects on endothelial cells, leukocytes, and keratinocytes [8].

The process of wound healing

Wound healing progresses through three distinct phases, inflammation, proliferation, and remodeling. These phases, which have considerable overlap, are somewhat theoretically artificial but provide a conceptual framework. It should also be emphasized that much of what we know about these overlapping healing phases come from experimental animal studies, and the findings are generally extrapolated to humans. The immediate tissue response to wounding is clot formation to stop bleeding. Simultaneously, there is a release of inflammatory cytokines that regulate blood flow to the area, and recruitment of lymphocytes and macrophages to fight infection. This initial inflammatory phase stimulates angiogenesis, fibroblast proliferation, and collagen deposition [8], which marks the proliferation phase. Fibroblasts rich in smooth muscle actin, called myofibroblasts [9], are recruited through the action of factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β; generally the TGF-β1 isoform). These latter processes result in the formation of granulation tissue, a highly vascularized and cellular wound connective tissue. In the remodeling phase of healing, granulation tissue is gradually replaced by scar tissue through the action of the myofibroblasts and factors such as TGF-β. Keratinocytes are stimulated to proliferate and to migrate into the wound bed to restore epidermal coverage.

Impaired healing and its mechanisms

Although there have been many recent advances in our understanding of the scientific basis of tissue repair, the treatment of chronic wounds with impaired healing has been very challenging. The healing of chronic wounds is impaired for a number of reasons, and in general these types of wounds remain challenging because of our inability to completely correct their fundamental pathophysiological abnormalities [10]. Preparatory steps before the use of advanced treatments seem to be required; this preparatory phase may not have received proper attention in the past. This situation is slowly being corrected, but much more needs to be implemented in preparing the wound for the optimal success of advanced wound healing factors and bioengineered constructs. Several years ago, we proposed the notion of "wound bed preparation" (WBP) as a series of steps to improve the wound before advanced products are used [11]. This concept has gained acceptance (see later).

Acute versus chronic wound healing

One of the basic differences between acute and chronic wounds is that in the former the sequence of steps and phases involved (clot formation, inflammation, migration and proliferation, and remodeling) occurs in a very orderly and linear fashion. On must be fully aware of the fact that the often cited steps involved in wound healing come from observations in experimental animal wounds, generally in mice and rats. Therefore, at least theoretically, the steps may be significantly different in human wounds. Anyhow, the orderly steps involved in acute wounds are not the same as in chronic human wounds, where there is a fundamental asynchrony of the healing process. Within the chronic wound, the various phases of wound repair may be occurring at the same time, or not in the appropriate sequence. WBP is a way to get the chronic wound to behave more like an acute wound. Often, surgical debridement is all that is required. At other times, treatment of bacterial infection, removal of edema, etc., are essential additional steps [11].

Bacterial colonization

Bacterial and, in some cases, fungal colonization or infection is a fundamental problem with nonhealing wounds. Some of the causes that foster this colonization and the development of occult infection have already been addressed. These factors include absent epithelium and its barrier properties, the constant wound exudate resulting from bacterial products and inflammation, and poor blood flow and hypoxia [10]. It is well established that wounds have a "bacterial burden" that interferes with healing. Thus there is evidence that, regardless of the type of bacteria present, a level greater than or equal to 10^6 organisms per gram of tissue is associated with serious healing impairment [12]. The configuration of the bacterial sheets or colonies within wounds is also important. Therefore there is a great deal of interest at this time in the role played by biofilms, which represent bacterial colonies surrounded by a protective coat of

polysaccharides. Biofilms develop mechanisms for antibiotic resistance [13].

Growth factor imbalances

The poor healing response in chronic wounds has also been attributed to an imbalance of one or more growth factors [14]. Identification of putative wound healing factors has led to several attempts to speed wound healing by local application of one or more factors that promote cell attachment and migration. However, most if not all, growth factor–based approaches have had marginal success. TGF-β, epidermal growth factor, vascular endothelial growth factor (VEGF), and PDGF have been candidates for this purpose [15–19]. Of these, only PDGF has shown efficacy in clinical trials and has FDA approval for clinical use (Regranex, HealthPoint Biotherapeutics Ltd., Ft. Worth, TX).

The arginine–glycine–aspartic acid (RGD) matrix peptide sequence has been found to promote the migration of connective tissue cells and thus stimulate production of a dermal scaffold within the wound bed. This approach has been shown to accelerate healing of sickle-cell leg ulcers [20] and diabetic ulcers [21], compared with placebo, but not when compared with standard care.

Complex cell extracts have been used in hopes of providing the appropriate mixture of elements. These include the use of platelet extract to provide primarily PDGF [22] and other stimulatory factors, and the use of conditioned growth medium to provide a complex mixture of elements derived from rapidly growing keratinocytes [23]. Again, these approaches have met with marginal effects, in part due to the complex nature of the wound healing response [24]. In addition, the use of factors is not a sufficient approach, in and of itself, in situations where there is severe or massive loss of skin tissue.

Matrix metalloproteinase activity

The nonhealing wound microenvironment can best be described as hostile. Wound exudate, in general, the vast abnormalities in the release, activation, and persistence of matrix metalloproteinases (MMPs), lack of cell adhesion to substrates within the wound bed all may render the growth factors and cytokines unavailable to the healing process. A concept that takes into account these various components has been called the "trap hypothesis" [25]. It has been hypothesized that nonhealing wounds, particularly in response to bacterial antigens, are characterized by chronic leakage of macromolecules into the wound. These macromolecules may impair healing by "trapping" cytokines and growth factors. The trap hypothesis suggests that, in spite of achieving adequate levels and even the properly orchestrated release of these growth factors, the polypeptides are quickly bound and unavailable to the healing process. Common macromolecules that might be involved in trapping include albumin, fibrinogen, and α-2-macroglobulin. The latter is particularly important because it is an established scavenger for growth factors. Fibrinogen can bind to fibronectin, providing a mechanism for the trapping of TGF-β1 [26].

Moist wound healing in chronic wounds

Moist wound healing has been shown experimentally to accelerate reepithelialization of acute wounds [27], and these observations have led to a number of moisture retentive dressings [28]. For chronic wounds, moist wound healing has not been clearly shown to improve epidermal healing or resurfacing, but we do know that moist wound healing helps in the formation of granulation tissue and in relieving pain. Painless debridement, too, is another important property of moisture retentive dressings [28]. The properties of moist wound healing are important in the field of bioengineered skin constructs, because such constructs lead to increased moisture in the wound bed. Proposed advantages of moist wound healing include retention of cytokines within the wound, general prevention of wound crust and facilitation of keratinocyte migration, prevention of bacterial entry, and even poorly understood but favorable electrical gradients. For example, acute wound fluid stimulates the in vitro proliferation of fibroblasts, keratinocytes, and endothelial cells. However, fluid and exudate from chronic wounds appear to have a definite adverse effect on cellular proliferation and contain excessive amounts of MMPs, which can break down key matrix proteins critical to cell migration, such as fibronectin and vitronectin [10]. There is a great deal of information we still need about MMPs and their inappropriate activation in chronic wounds. Some of the information is often contradictory. For example, interstitial collagenase (MMP-1) is essential for keratinocyte migration. However, other enzymes (MMP-2 and MMP-9) may prevent or interfere with healing [10].

Ischemia

An ultimate goal for tissue engineering constructs would be to offset very fundamental abnormalities that lead to impaired or slow healing. We have been discussing components of impaired healing that in some way or another can be approached or at least partially corrected by already available means. However, one important component of impaired healing is ischemia, due to poor arterial supply because of narrowing of blood vessels (i.e., atherosclerosis) or, indirectly, because of pressure upon those blood vessels (i.e., pressure ulcers and diabetic

neuropathic ulcers). The ischemia, of course, has important consequences for the other components of impaired healing we have discussed, such as bacterial colonization and infection. A challenge is how to use available tissue engineering products or modify and develop new ones to correct the problem of ischemia. There are some interesting possibilities that one can use as proof of principle. An example is the role of oxygen tension. Thus there is no debate over the fact that long-term hypoxia is detrimental to the healing process. This has been readily shown with diabetic ulcers, where low levels of transcutaneous oxygen tension ($TcPO_2$) correlate with inability to heal [29]. However, as this makes sense even from a teleological point of view, short-term hypoxia actually stimulates healing. It has been shown that hypoxia can increase fibroblast proliferation, fibroblast clonal growth, and the synthesis of several growth factors, including PDGF, TGF-β, and VEGF [10]. Therefore modulation of the oxygen environment within the wound may offer the possibility of accelerating the healing process. Hypoxia-inducible transcription factors represent now well-established mechanisms for how cells sense hypoxia.

Abnormalities at the cellular level

A very important mechanism for impaired healing is the phenotypic makeup of wound cells. This has critical implications for the use of bioengineered skin constructs, in that these constructs may offset cellular phenotypic abnormalities. There is increasing evidence that the resident cells of chronic wounds have developed phenotypic changes that interfere with their response to growth factors and cytokines. Such abnormalities may affect cellular proliferation, locomotion, and the overall capacity to heal [10]. Also, the signaling mechanisms, which are so critical to the action of cytokines, may be impaired. For example, at least in venous ulcers, there is decreased phosphorylation of Smad2/3 and MAPK p44/42 [30], and a complete deregulation of keratinocyte differentiation and activation and therefore wound closure [31], a result of impaired TGF-β signaling [32].

Engineering skin tissue

Although the epidermis has an enormous capacity to heal, there are situations in which it is necessary to replace large areas of epidermis, or in which normal epidermal regeneration is deficient. The dermis has very little capacity to regenerate. The scar tissue that forms in the absence of dermis lacks the elasticity, flexibility, and strength of normal dermis. Consequently, scar tissue limits movement, causes pain, and is cosmetically undesirable. Engineered tissues that not only heal wounds but also stimulate the regeneration of dermis would provide a significant benefit in wound healing.

Bioengineered skin equivalents should incorporate as many of these factors as possible:

1. an ECM;
2. dermal fibroblasts;
3. an epidermis containing keratinocytes; and
4. a naturally occurring semipermeable membrane, the stratum corneum to provide barrier function (Fig. 71.2).

These components may act alone, but more importantly and likely, they act in concert as part of a fully integrated tissue to protect the underlying tissues of a wound bed and to direct healing of the wound [33]. Dermis containing fibroblasts may be necessary for the maintenance of the epidermal cell population [34]. In turn, the epidermis is necessary for the formation of the so-called neodermis, in the absence of a dermal layer [35], and can dramatically influence underlying connective tissue responses. The formation of the epidermal barrier also likely influences these processes through control of epidermal water loss and its influence on epidermal physiology [36].

Design considerations

Tissue engineering has not focused on regenerating certain skin structures, such as hair follicles or sebaceous glands, loss of which is clinically less significant than the loss of dermis and epidermis needed to cover and protect the

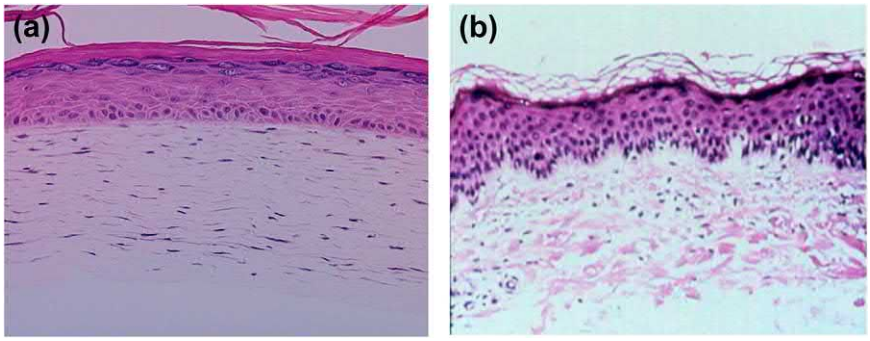

FIGURE 71.2 Histological appearance of Apligraf.
(A) Compared to normal human skin. (B) A well-defined epidermal and dermal layer is observed in both. *V. Falanga, ©2006.*

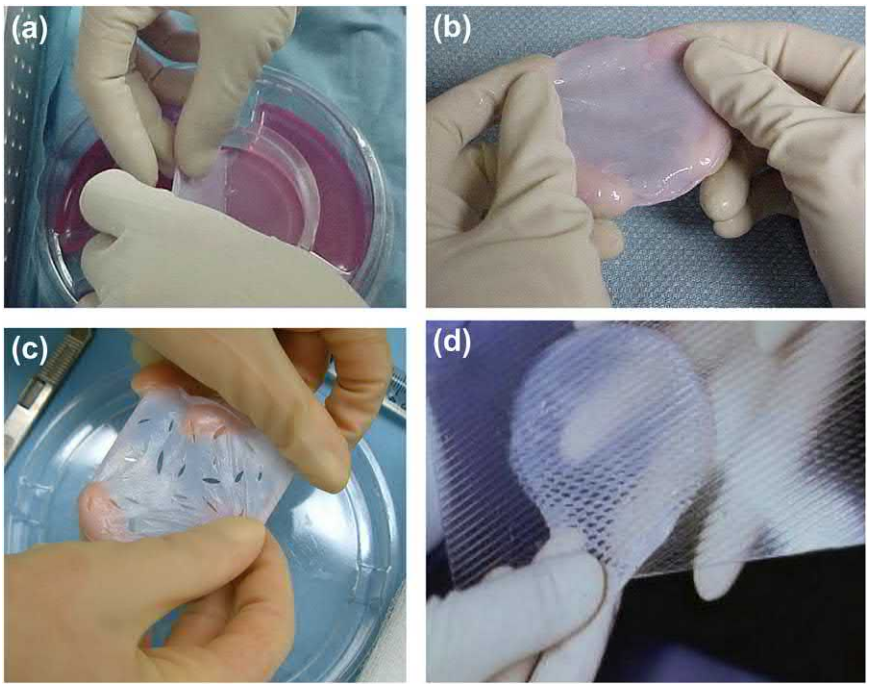

FIGURE 71.3 Appearance of Apligraf. (A) Being removed from its pink nutrient agar. (B) Being held just prior to fenestration or meshing. (C) After fenestration with a scalpel. (D) After meshing at a 1.5:1 ratio. *V. Falanga, ©2006.*

underlying tissues. Despite some early evidence for rudimentary eccrine tubules within the HSE [37], the full development of functioning adnexal structures is likely to be several years away, although progress is being made. There has also been little need to extraneously stimulate regeneration of other dermal components (e.g., blood vessels and cells of the immune system) through tissue engineering methods because these components have the ability to repopulate quickly and to normalize the area of a wound. Langerhans cells, for example, have been shown to migrate and repopulate effectively within months [9]. Control of vascularization is dependent on the makeup of the ECM and the degree of inflammation present in the wound. Whether modification of vascularization through the use of exogenous factors will be of additional benefit for certain wounds still remains to be determined. While pigmentation is not critical for wound healing, as far as we can determine at this time, clinical studies using Apligraf, which lacks melanocytes, have shown repigmentation of the grafted areas through repopulation of the area with host melanocytes, resulting in normal skin color for many patients. The constructs should have sufficient mechanical strength to allow for clinical manipulations (Fig. 71.3). The approach skin tissue engineering has taken has been to focus primarily on providing or imitating structural and biological characteristics of dermis, epidermis, or both.

Following are the key features to be replicated in an engineered skin construct:

- a dermal element capable of aiding appropriate dermal repair and epidermal support;
- an epidermis capable of easily achieving biologic wound closure;
- an epidermis capable of rapid reestablishment of barrier properties;
- a permissive milieu for the components of the immune system, nervous system, and vasculature;
- a tissue capable of achieving normalization of structure and additional functions such as reduction of long-term scarring and reestablishment of pigmentation;
- active cellular component(s) capable of responding to different wound types and conditions;
- sufficient mechanical strength to allow for clinical manipulation; and
- persistence of cells in the wound for multiple weeks to stimulate the healing process through delivery of cytokines and matrix proteins.

Commercial considerations

Engineered skin constructs, by virtue of being the first bioengineered products to be commercialized, have been at the forefront of science, industry, and regulation. In recent years, more attention has been given to the subject of their commercialization. Jaklenec et al. published a review of the tissue engineering and stem cell industry [38]. The authors reported that there was approximately a threefold increase in commercial sales, generating $3.5 billion from 2008 to 2011. During that time the number of tissue engineering companies increased from 171 in 2007 to 202

in 2011, with 62% of them based in the United States. These companies are spending $3.6 billion and employ 13,810 people. The findings indicate that the tissue engineering and stem cell industries are stabilizing.

Early industry leaders, Organogenesis, Advanced BioHealing (now Shire Regenerative Medicine) and Forticell Biosciences, initially struggled with the task of commercializing their technologies. The focus at Organogenesis, in the case of Apligraf, has been on understanding the cost of manufacturing and working to reduce those costs while increasing efficiency and maintaining high quality. Organogenesis has also built robust sales and marketing, customer service, reimbursement, and distribution functions to drive Apligraf sales. Having all of these functions within the same organization is proving to be of benefit by allowing for a more integrated, strategic company.

Process considerations

Consideration should be given to the design of the manufacturing process early in the product concept stage. Elements such as critical process parameters, quality control assays, production components and materials, process equipment, production facility requirements, product's shelf life, and distribution methods need to be developed in detail. From this design the cost of manufacturing and strategies for scale-up and automation can be developed. The requirements for the manufacturing facility are directly related to the design of the manufacturing process. For example, a manual process generally requires a large highly specialized staff and a relatively large manufacturing floor space, whereas an automated process requires less specialized staff and potentially less manufacturing floor space. An aseptic process where the culture vessels are periodically opened to facilitate feeding and other manipulations requires tighter environmental controls to mitigate risk from contaminants on work surfaces or in the air, whereas developing a closed process that eliminates aseptic manipulations reduces the need for tight environmental controls, thus reducing facility costs. All of these elements impact the cost of manufacturing and the potential for scale-up.

Process components that will pose significant barriers to scale-up or automation can be identified and addressed while the product is still in research and development. This is critical when you consider that the further you advance in the development process the more committed you become to the process design. The cost of making a significant process change increases the closer you get to manufacturing and becomes exponentially more expensive and time-consuming after product launch. Along these same lines, materials used in the process that are ill-defined, cell-based, single sources or of limited supply can contribute to a manufacturing process that is difficult to scale-up and control from a quality standpoint. These factors will influence the yield from the manufacturing process or, stated another way, the scrap rate for the product. High scrap rates increase manufacturing costs and create issues for inventory management. The pioneering lessons learned by early industry leaders in process scale-up apply to the broader field of tissue engineering. Ultimately, strategies for scale-up and automation can be evaluated and planned for.

Regulatory considerations

The Center for Devices and Radiological Health at FDA requires products to be developed following design control procedures (21 CFR 820.30). These design guidelines are in place to ensure that the products that are developed will be safe, effective and have a market research–based justification for the product design. Establishing a design control system early will help refine the business model throughout the development life cycle. Design control is not only a regulatory requirement, but also it makes good business sense. HSEs have historically been classified and regulated as medical devices, though the trend at FDA is to regulate products having a cellular component as biologics. A case in point is the recent approval of GINTUIT, a fibroblast- and keratinocyte-containing construct, as a combination product (containing biologic and devices components) by the Center for Biologics Evaluation and Research (CBER) [39]. Finally, agencies such as the US Center for Medicare and Medicaid Services and foreign government reimbursement agencies (e.g., UK National Institute for Health & Clinical Excellence) are increasingly requiring convincing data on the value and effectiveness of therapy as compared to standard of care or competing technologies [40].

It should be noted that, when it comes to academic investigators (typically in a University Medical Center) developing novel and experimental bioengineered skin constructs, a new reality has developed in the last several years with regard to regulatory requirements. It used to be that regulatory agencies, such as the FDA, may not have held the noncommercial product development with the same stringency and requirements. It is now well established the good manufacturing practice facilities are required. This degree of stringency now also extends to the evaluation of National Institutes of Health (NIH) awards for new and novel projects. Thus, within the NIH, committees have been established, which collaborate and communicate with federal regulatory agencies for absolute safety of the products being developed and tested.

Immunological considerations

The ability to utilize allogeneic cells rather than autologous cells facilitates the reproducible, large-scale commercial manufacture of an HSE [37], because large cell banks can be created, allowing manufacture of thousands of units of product from one or few comparable cell strains and enabling a more accurate forecast of manufacturing demands. However, the problem of rejection needs to be taken into consideration when using living allogeneic cells in wound healing applications.

The first stage in the induction of a primary immune response after skin allografting is the presentation of antigen by donor dendritic cells (Langerhans cells with dermal dendritic cells), the professional antigen-presenting cells (APCs) in skin. These cells migrate out of the skin to the draining lymph node, where they can activate T cells directly through the presentation of MHC-class II antigens and costimulatory molecules, thereby eliciting both cell-mediated and humoral (antibody-mediated) immune responses to the grafted skin. Although cell-mediated cytotoxicity is a component of rejection, the primary mode of skin rejection is likely mediated via an attack on the vasculature present in a normal skin graft by recipient antibodies [41,42].

For the most part, bioengineered skin constructs are fabricated from highly purified banks of fibroblasts, keratinocytes, or both, which are either poor or deficient in dendritic and other APCs found in skin. This has important implications for the use of allogeneic cells in the treatment of acute and chronic wounds. In the absence of APCs, fibroblasts and keratinocytes are the only cells capable of presenting donor antigen to the recipient. Under normal conditions, keratinocytes and fibroblasts do not express MHC-class II antigens. They can be induced by interferon-γ to express MHC-class II molecules and thereby acquire the ability to present antigen to T cells; however, keratinocytes and fibroblasts do not express costimulatory molecules [43,44], so antigen presentation by keratinocytes and fibroblasts does not result in T cell activation. Instead, this antigen presentation can result in T cell nonresponsiveness [45,46] or T cell anergy [47]. It should be noted that there are continued efforts to develop, test, and commercialize constructs comprising also endothelial cells and/or adnexal skin structures. Therefore the abovementioned immunological aspects will need to be considered when creating and testing even more complex constructs.

Autologous HSEs would avoid issues of immunogenicity, of course, but autologous grafts have significant limitations. The process of growing constructs' components from skin biopsies takes several weeks, the donor site creates another wound and, in some patients (e.g., severe burn victims), there may be no appropriate and safe donor site. Reproducibly making complex HSE constructs to order from autologous cells would be time-consuming, and very costly. Therefore the ability to effectively use allogeneic human cells is a key element in the commercial success of engineered skin therapies.

Summary: engineering skin tissue

In summary, good science alone is not enough to ensure success. There are critical considerations to commercialization along the entire continuum from product concept, development, clinical evaluation, FDA and other regulatory agency approval, product launch, reimbursement, and commercial-scale manufacturing. Developing a comprehensive business model that takes all of these factors into account will increase the likelihood for a profit-generating product. Understanding the challenges and working to incorporate process designs that are forward-looking, will be amenable to scale-up, address regulatory hurdles, and are supported by a viable business model are necessary for companies to be successful and the industry to grow.

Epidermal regeneration

Reepithelialization of the wound is a paramount concern. Without epithelial coverage, no defense exists against contamination of the exposed underlying tissue or loss of fluid. The approaches to reestablishing epidermis are numerous, ranging from the use of cell suspensions to full-thickness skin equivalents possessing a differentiated epidermis. Silicone membranes have been used as temporary coverings in conjunction with dermal templates [48], porcine skin and cadaver skin have been used in burn victims. Regardless of approach, living epidermal keratinocytes are necessary to achieve permanent, biologically based wound closure.

Green et al. [49,50] developed techniques for growing human epidermal keratinocytes from small patient biopsy samples using coculture methods [51]. The mouse 3T3 fibroblast feeder cell system allows substantial expansion of epidermal keratinocytes and can be used to generate enough thin, multilayered epidermal sheets to resurface the body of a severely burned patient [52]. Once transplanted, the epidermal sheets quickly form more differentiated epidermis and reestablish epidermal coverage [35]. With time, the cultured epithelial autograft (CEA) stimulates the formation of new connective tissue (neodermis) immediately beneath the epidermis [53], but scarring and wound contraction remain significant problems [54]. Studies have shown that grafting of a CEA onto pregrafted cadaver dermis greatly improves graft take [55]. CEAs (Epicel, Genzyme, Cambridge, MA) have been available since the late 1980s.

Dermal replacement

Dermal replacement is an essential first step in restoring normal skin structure following injury and leads to better cosmesis in full-thickness soft tissue defects. In one of the earliest tissue engineering approaches to improving dermal healing, Yannas et al. [56] designed a collagen-glycosaminoglycan sponge to serve as a scaffold or template for dermal ECM. The goal was to promote fibroblast repopulation in a controlled way that would decrease scarring and wound contraction. A commercial version of this material composed of bovine collagen and chondroitin sulfate, with a silicone membrane covering (Integra, Integra Life Sciences, Plainsboro, NJ) has received regulatory approval for use in burns [48,57]. The dermal layer is slowly resorbed, and the silicone membrane is eventually removed, to be replaced by a thin autograft [58].

Biobrane is a nonviable temporary covering for burns, in which a nylon mesh, coated with porcine collagen−derived peptides and layered with a nonpermeable silicone membrane serves as a platform for deposition of human matrix proteins and associated factors by host wound−derived dermal fibroblasts. After epidermalization the construct becomes nonadherent and is removed from the wound bed. A similar product, Transcyte (Advanced BioHealing, Westport, CT) [59], consists of a porcine collagen−coated nylon mesh that is incubated with human neonatal fibroblasts, which secrete ECM proteins and growth factors. The material is then frozen to preserve the matrix and factors produced by the fibroblasts, although the fibroblasts themselves are nonviable when the construct is applied to the wound. The temporary silicone covering is removed to allow epidermalization or prior to application of an autograft.

Although matrix scaffolds have shown some improvement in scar morphology, no acellular matrix has yet been shown to lead to true dermal regeneration. This may be due in part to limits in cell repopulation, the type of fibroblast repopulating the graft, and control of the inflammatory and remodeling processes (i.e., the ability of the cells to degrade old matrix while synthesizing new matrix).

The inflammatory response must be controlled in dermal repair in order to avoid the formation of scar tissue. Therefore dermal scaffolds must not be inflammatory and must not stimulate a foreign body reaction. This has been a problem in the past for some glutaraldehyde cross-linked collagen substrates [60]. The ability of the matrix to persist long enough to redirect tissue formation must be balanced with effects of the matrix on inflammatory processes. One way to achieve this is to form a biological tissue that is recognized as living tissue, not a foreign substance.

There have been advances in the design of in vitro grown dermal tissues using living human neonatal fibroblasts grown on rectangular sheets of biodegradable vicryl mesh (Dermagraft, Shire Regenerative Medicine, San Diego, CA). The fibroblasts propagate among the fibers of the mesh, producing ECM in its interstices [61]. Like Transcyte, the final Dermagraft construct is then frozen, but in a manner that preserves approximately 60% fibroblast viability upon thawing and placement on the wound [62].

Bioengineered living skin equivalents

HSEs have been generated by seeding cultured keratinocytes onto the surface of a variety of scaffolds, including artificial membranes, deepidermalized dermis, collagen gels, chitosan-GAG sponges, or fibroblast-populated nylon meshes [63]. One of the first attempts to replicate a full-thickness skin graft was by Bell et al. [64] who described a bilayered skin equivalent, which was the predecessor to Apligraf (Organogenesis, Canton, MA). The dermal component consisted of a lattice of type I collagen contracted by tractional forces of rat dermal fibroblasts trapped within the gelled collagen. This contracted lattice was then used alone or as a substrate for rat epidermal keratinocytes. These primitive skin equivalents were further demonstrated to take as skin grafts in rats. This technology has now advanced to enable the production of large amounts of the Apligraf construct from a single donor [37].

Boyce et al. modified the approach first proposed by Yannas et al. to form a bilayered composite skin by using a modified collagen-glycosaminoglycan substrate seeded with fibroblasts and overlaid with epidermal keratinocytes [65]. An autologous form of this composite skin construct was used to treat severe burns with some success [66]. An allogeneic form of the construct showed improved healing in a pilot study in chronic wounds [67]. A similar technology has been studied in the treatment of patients with genetic blistering diseases [68]. Using methods of organotypic culture that provides a three-dimensional culture environment that is permissive for proper tissue differentiation, the resulting HSE develops many of the structural, biochemical, and functional properties of human skin [65,69−71], apart from the aforementioned lack of adnexal structures.

The process for the formation of HSEs has been covered in detail many times [37,65,72] and will not be detailed here. However, there are points to be made about the approach to these procedures. The culture of an HSE proceeds best with minimal intervention. Normal keratinocyte populations seem to have an intrinsic ability to reexpress their differentiation program in vitro. A medium that supplies adequate amounts of nutrients, lipid precursors, vitamins, and minerals may be all that is required [37]. This has now been achieved with both normal human keratinocytes (Apligraf, Orcel) and with immortalized human keratinocytes (StrataGraft) [73]. Another

element is the environmental stimulus provided by culture at the air–liquid interface, which promotes differentiation and formation of the epidermal barrier [65,69]. In the case of StrataGraft the trisomy that caused immortalization of the cell line has apparently not affected the capacity of the cells to undergo differentiation in organotypic culture [73].

L'Heureux et al. were able to take advantage of the fact that mesenchymal cells secrete their own ECM when cultured in the presence of ascorbic acid, to generate sheets of ECM, in the absence of a bovine collagen scaffold, from cultured fibroblasts and smooth muscle cells. Initially, these ECM tissues were used to generate the first tissue-engineered blood vessels [74]. Subsequently, the same technology was used to generate a scaffold-free HSE [75], in which the dermal layer normally constructed with bovine collagen was replaced with a completely fibroblast-derived ECM. The epidermis of the HSE was generated by seeding keratinocytes onto the self-assembled ECM and incubating at the air–liquid interface to promote stratification and differentiation. After 4 weeks of culture a fully stratified and differentiated epithelium developed, which expressed the normal markers of differentiation. The tissue also included an organized basement membrane, including laminin and collagens IV and VII. Since this early work a few groups have investigated the physical and biochemical properties of self-assembled ECMs [75–77] and have used these self-assembled constructs as in vitro models of percutaneous absorption [75] and hypertrophic scarring [78,79]. However, self-assembled HSEs have yet to become a clinical and commercial reality.

Bioengineered skin: FDA-approved indications

Cutaneous indications

As discussed earlier, the rather formidable obstacles to healing chronic wounds have made it difficult for the simple topical application of growth factors and cytokines to have a successful outcome. It can be argued that tissue engineering, particularly with living cells, has an advantage in that cells may be capable of responding to the microenvironment and thus behave in a "smart" way from an engineering point of view. Because of these considerations, cell therapy with bioengineered skin has been tested in difficult to heal wounds. Figs. 71.2 and 71.3 show Apligraf before and after meshing and fenestration, as well as its histological appearance. Venous leg ulcers (VLUs) and neuropathic diabetic foot ulcers (DFUs) have received the greatest attention. Two main types of living bioengineered skin have been proven to be effective in diabetic neuropathic foot ulcers and have received regulatory approval from the FDA. In a randomized 12-week trial of 208 patients with neuropathic ulcers, Apligraf led to complete wound closure in 56% of patients, compared to 38% in the control group [80]. The Kaplan–Meier median time to complete wound closure was 65 days for Apligraf and 90 days for control. Fig. 71.4 shows a

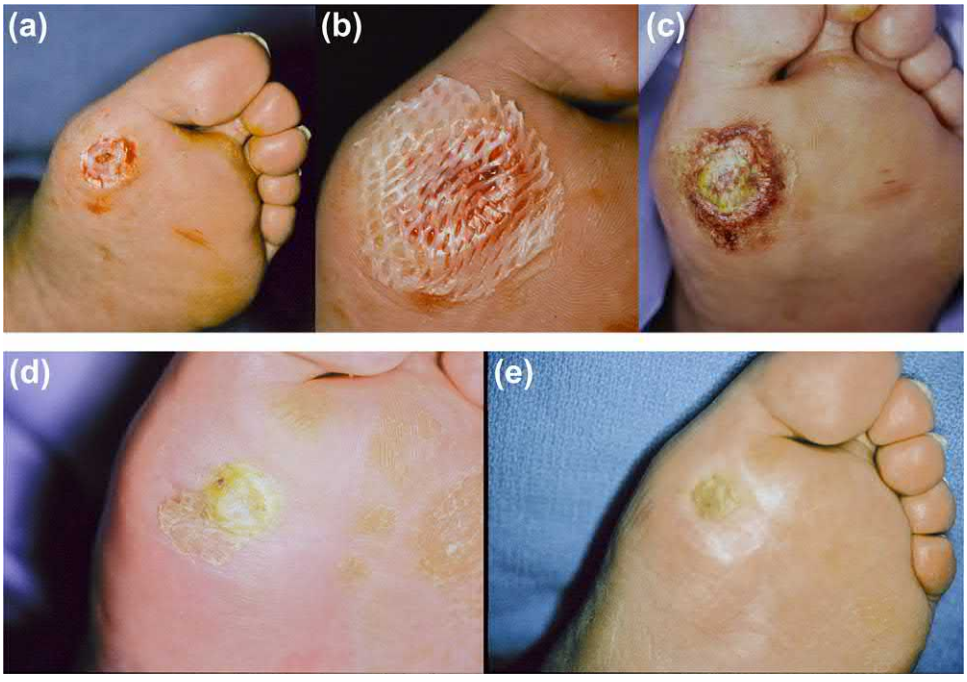

FIGURE 71.4 Diabetic neuropathic foot ulcer successfully treated with Apligraf.
(A) Just after surgical debridement to remove the necrotic wound bed and surrounding callus. (B) Wound covered with meshed Apligraf. (C) Appearance of construct a week later. (D) At 3 weeks. (E) Complete wound closure at 5 weeks. *V. Falanga, ©2006.*

diabetic neuropathic foot ulcer successfully treated with Apligraf. In an evaluation of the clinical trial adverse events, Apligraf treated patients had significantly fewer amputations/resections of the study limb and less incidence of osteomyelitis. These results on key complications of DFUs may have been due to faster healing. Another living cell product, Dermagraft, was shown in a 12-week randomized study to heal neuropathic foot ulcers, with an incidence of closure of 30% and 18% for the active and control arm, respectively [81]. It is perplexing that the rate of healing in the control was widely different between Apligraf (38%) and Dermagraft (18%), but no definite explanation has surfaced for that discrepancy.

In addition to its indication for diabetic ulcers, Apligraf also remains the only approved bioengineered product for venous ulcers. In a pivotal multicenter randomized study of 293 patients with VLUs, Apligraf was more effective than leg compression alone (control) in the percentage of patients who healed by 6 months (63% vs 49%) [82]. That study also showed that Apligraf healed ulcers, which were larger and deeper than those in the control group. No evidence of sensitization was observed with the bioengineered skin product. Interestingly, a subsequent reanalysis of the data from this pivotal trial, limited to 120 patients having venous ulcers for over 1 year, showed that Apligraf healed 47% of patients, compared to 19% in the control group [83]. This observation, taken together with the mechanisms underlying impaired healing, suggests that cell therapy might have a greater effect when there are more substantial abnormalities leading to a prolonged failure to heal.

Oral indications

Like skin, soft tissues in the oral cavity need to be replaced or repaired after physical/surgical trauma, disease processes, or congenital defects. Successful treatment of soft tissue defects in the oral cavity has been accomplished by either secondary intention healing (removing the diseased tissue and allowing it to heal on its own), by the use of flaps (a graft that is advanced over a defect and contains its own blood supply), or by the use of free grafts (tissue that lacks its own blood supply). Depending on the procedure, auto- or allografts may be used. Autografts are generally harvested from the palate and are associated with significant donor site morbidity. However, some groups have had success with autologous epithelial cells, and with injected autologous mucosal fibroblasts [84].

Two types of allogeneic, tissue-engineered constructs have been used in the oral cavity. Dermagraft has been used in the oral cavity to treat gingival recessions, in which the amount of attached gingiva was insufficient. As with its DFU indication, it is thought that the Dermagraft construct provides a scaffold for mucosal keratinocyte migration, and that it secretes growth factors and cytokines that promote secondary intention healing. Dermagraft has been the subject of a controlled within-patient clinical trial in the oral cavity, in which its efficacy was compared to that of a gingival autograft for the treatment of gingival recessions [85,86]. Treatment with Dermagraft resulted in an increase in the amount of attached gingiva, although the amount of keratinized tissue was inferior to that generated by the autograft, as expected from a construct that promotes secondary intention healing rather than immediate coverage.

GINTUIT, a product derived using a similar technology platform as Apligraf, has also been evaluated for its ability to replace a connective tissue autograft in the treatment of gingival recessions in a free gingival graft procedure [87]. Like Apligraf in the DFU and VLU indications, GINTUIT did not persist long-term in the oral cavity. Like Dermagraft, GINTUIT was found to be superior to a gingival autograft in terms of color and texture match to surrounding tissue. In the pilot and pivotal studies, treatment with GINTUIT resulted in regeneration of ≥ 2 mm of keratinized gingiva in 81.8% and 95.3% of patients, respectively, which is clinically accepted as a level important for periodontal health. GINTUIT was the first living cell–based therapy that has FDA approval for the treatment of oral soft tissue defects [39]. Although derived from the same technology platform as Apligraf, GINTUIT was approved as a combination product (containing biologic and devices components) by the CBER [39] and is therefore subjected to additional product release criteria compared to Apligraf. Interestingly, the platform that gives rise to Apligraf and GINTUIT is able to regenerate tissue in a site-appropriate manner, in that it promotes secondary intention healing of difficult to heal cutaneous soft tissue defects, and also promotes regeneration of keratinized gingiva site-appropriately in the oral cavity.

Apligraf and Dermagraft: off-label uses

Off-label use is the practice of using products for purposes not approved by the FDA. Physicians can prescribe and use products as they see fit because they are not regulated and are recognized as experts in their fields. Manufacturers of the products are not permitted to promote off-label use; however, data generated by off-label use can be very valuable and lead to new insights into product function and potentially to clinical trials for new indications, therefore the following discussion has been included in this chapter. It must be emphasized, however, that the determination of off-label use is not always clear-cut when it comes to chronic wound etiology. In some cases applicable to Apligraf, for example, a scalp or hand

wound, one is clearly using the product off-label because that construct is FDA approved for venous and diabetic neuropathic foot ulcers. However, for many lower extremity wounds, the situation is much more complex. Thus a patient may have a mixed etiology for the ulcer (e.g., lymphatic and venous insufficiency) and will therefore not heal until the venous component is properly addressed. In addition to the use of standard care for venous insufficiency, such as compression bandages and wound dressings, one could make the argument that Apligraf is indicated in that clinical setting because the construct has indeed been proven to improve venous ulcers. It would be difficult, perhaps impossible, to conduct clinical studies that address ulcers of mixed etiology in a scientifically sound way. An even more complex clinical setting is when one treats patients who appear to have inflammatory ulcers (i.e., pyoderma gangrenosum, ulcers due to or associated with vasculitis and cryoprotenemias) on a lower extremity that also exhibits definite signs and findings of venous insufficiency. The question then centers upon the predominant etiology of the ulceration. From a clinical standpoint, it can become very difficult to make that determination. Yet treatment of that ulcer with Apligraf to improve the venous component can lead to complete wound closure (V. Falanga, personal observation as of 2019). These are challenging situations, but, in our experience and exchanges with colleagues, these types of ulcers are probably not uncommon. Still, while being able to use certain constructs off-label, the clinician must keep in mind the appropriate approved indications for that product and not be swayed in his or her clinical judgment as to what is indeed the primary cause of the ulcer.

From both a therapeutic and purely scientific standpoint, of critical importance in the context of the clinical trials for venous leg and DFUs that have been detailed is that, for the very first time, one has been able to show a beneficial effect of bioengineered products in situations of impaired healing. Understandably, clinicians have also used Apligraf off-label to accelerate healing in wounds not due to venous disease or diabetes [88]. Wounds that have been treated off-label with Apligraf include acute wounds after extensive skin surgery, donor sites after split-thickness skin harvesting, traumatic wounds, burns, inflammatory ulcers such as pyoderma gangrenosum and vasculitis, scleroderma digital ulcers, wounds after keloid removal, genetic conditions such as epidermolysis bullosa (EB), and a variety of situations that defy proper diagnosis because of their complexity. In these cases, bioengineered skin treatment with Apligraf seems to offer a viable alternative to stimulate wound healing and relieve unacceptable pain and suffering. For example, pyoderma gangrenosum is an inflammatory ulcer often associated with rheumatoid arthritis, inflammatory bowel disease, IgA gammopathy, among other predisposing factors. A peculiar feature of pyoderma gangrenosum is that it worsens with extensive surgical manipulation and even develops at distant and normal looking skin that are traumatized; thus autologous grafting is a contraindication, and bioengineered skin is an attractive therapeutic modality [89]. In a patient with multiple myeloma undergoing conditioning for bone marrow transplantation, extravasation of the chemotherapeutic drug adriamycin occurred in his chest and created a very large, deep, and painful wound. Apligraf played a major role in healing the full-thickness wound (Fig. 71.5) and led to a dramatic relief in pain. Pressure ulcers, too, have been treated with Apligraf. This is of particular interest because, based on the engineering aspects of the construct, one would have expected that very deep wounds with loss of a great deal of tissue would not respond to Apligraf. Yet it appears that in such cases Apligraf is able to stimulate tissue regrowth in deep ulcers, and not just reepithelialization from the edges of the wound (Fig. 71.6). Scleroderma digital ulcers are ischemic in nature and have no known accepted treatment. Yet Apligraf appears promising in those wounds (Fig. 71.7). There is no evidence that Apligraf stimulates malignancy within the wound bed, possibly due to its stimulatory action. An interesting case in point is shown in Fig. 71.8, where multiple and ulcerated basal cell carcinomas in the foot of a patient with the hereditary form of basal cell nevus syndrome were treated with Apligraf.

EB is a genetic skin disorder characterized by blistering of the skin and mucosae following mild mechanical trauma [90,91]. Falabella et al. performed an open-label uncontrolled study of 15 patents with EB treated with Apligraf [91]. The conclusion was that Apligraf induced rapid healing; the wounds remained healed for some weeks and there was no acute rejection reaction or other adverse effects related to the treatment. Currently, there is no specific treatment for EB, and the nature of the condition is that these wounds tend to heal slowly and in some instances fail to heal, becoming chronic wounds. Fig. 71.9 shows an example of a pediatric EB patient who was treated with Apligraf.

These considerations are not applicable to only Apligraf, although there has been more experience with that construct. A case of vasculitis treated with Dermagraft is another good example of the use of a tissue-engineered construct to promote closure of a difficult to heal wound (Fig. 71.10). The patient (48 years old) had a severe course of cutaneous polyarteritis nodosa, which did not involve her kidneys. She was always in severe pain and could not be healed by conventional means. Dermagraft proved useful in terms of alleviating pain and promoting epidermalization. Stimulation of epidermal migration was observed by Day 14 after

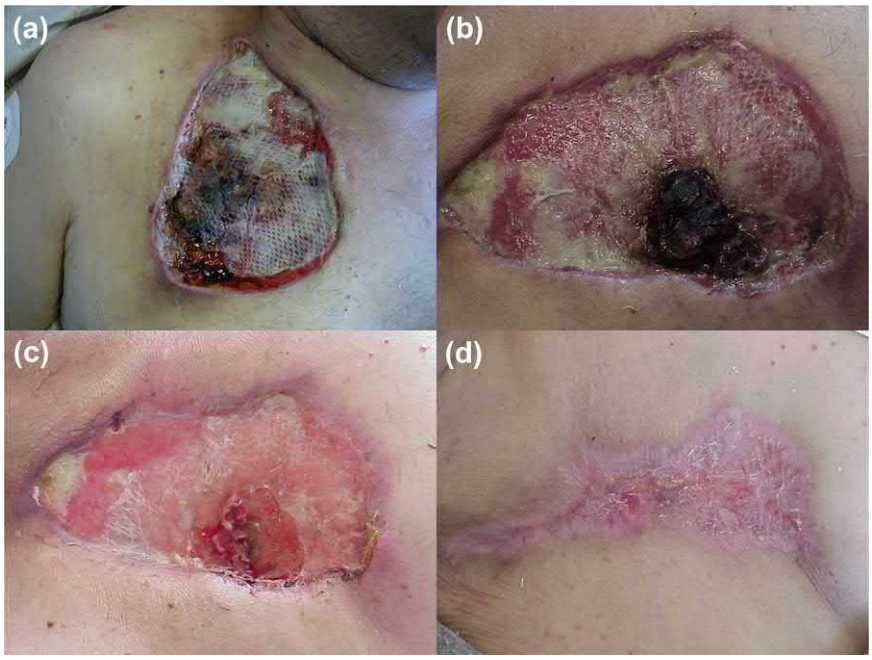

FIGURE 71.5 Large and deep wound from extravasation of adriamycin into the chest wall during treatment for multiple myeloma and bone marrow conditioning.
(A) Wound still partially covered with meshed Apligraf, 2 weeks after treatment with construct. (B) Wound at week 3. (C) Wound at 8 weeks. (D) Complete closure after 10 weeks. *V. Falanga, ©2006.*

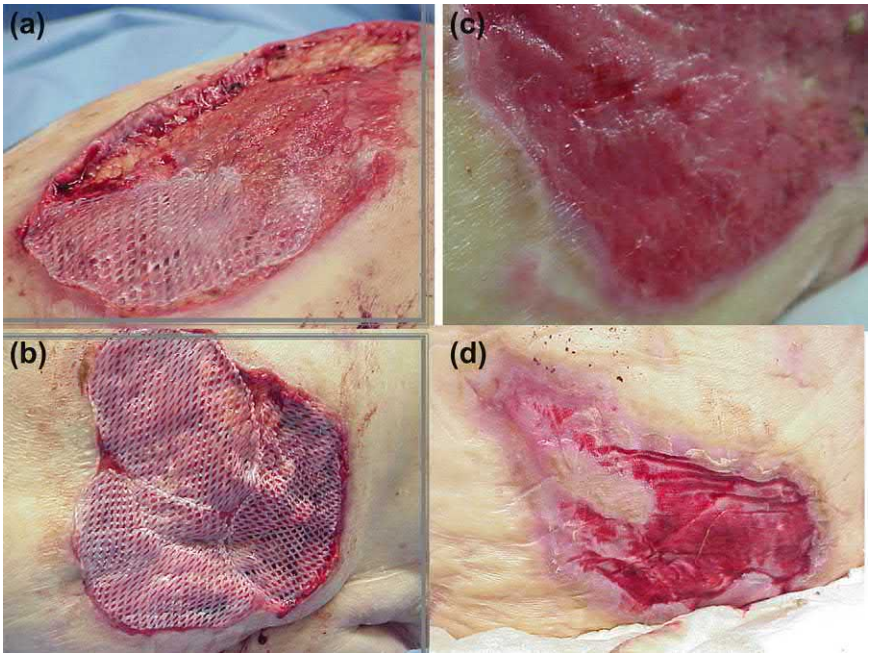

FIGURE 71.6 Large sacral pressure ulcer.
(A) Immediate after extensive surgical debridement and beginning of Apligraf application. (B) Wound fully covered with meshed Apligraf right after surgical debridement. (C) After 3 weeks the wound bed is now flush with the surrounding skin. (D) At week 5 the wound is largely epithelialized. The edges of the wound are rapidly advancing toward the center of the wound. *V. Falanga, ©2006.*

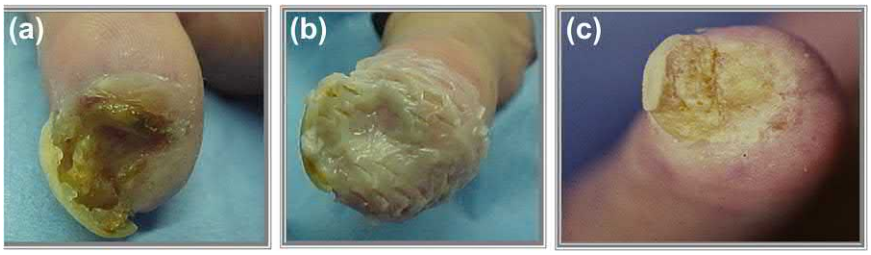

FIGURE 71.7 Systemic sclerosis (scleroderma) digital ulcer treated with Apligraf.
(A) Baseline ulcer with fibrinous wound bed. (B) Wound covered with meshed Apligraf. (C) Wound closure 4 weeks later. *V. Falanga, ©2006.*

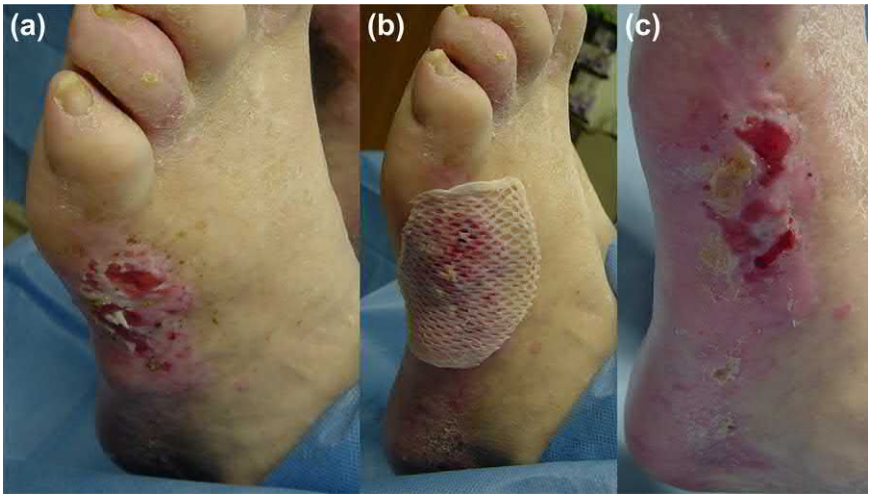

FIGURE 71.8 Patient with a hereditary form of basal cell carcinoma (the basal cells nevus syndrome). His entire body was covered with these skin cancers.
(A) Nonoperative ulcerated basal cell carcinomas (proven histologically) on the lateral side of the foot. (B) Ulcerated cancers treated with Apligraf. (C) Appearance of same site several months after Apligraf application and without obvious clinical evidence of extension of the skin cancers. *V. Falanga, ©2006.*

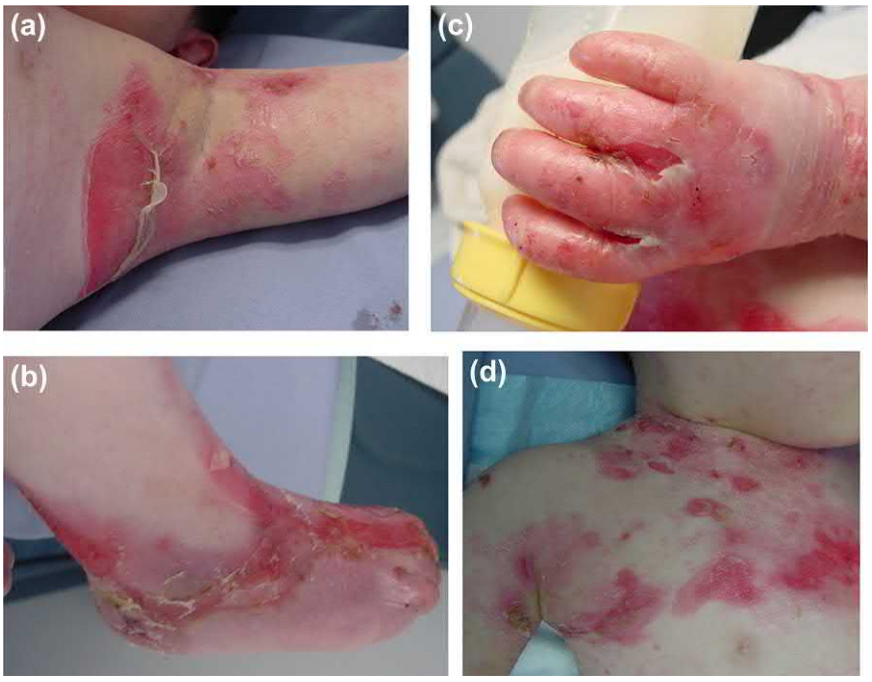

FIGURE 71.9 Epidermolysis bullosa (dystrophic type).
(A) Denuded area in axilla, with milia formation from repeated breakdown and healing. (B) Foot that has experienced many episodes of wounding, resulting in scarring and loss of toe webs. (C) Mitten deformity of the hand well under way due to constant injury and attempts at repair. (D) Several denuded areas on the chest, showing evidence of active epithelialization. The child had been treated with Apligraf. *V. Falanga, ©2006.*

application of the construct. After the wound healed the patient was placed on an anti-TNF-α biological agent to prevent recurrence and she remains healed.

The importance of wound bed preparation

Shortly after the FDA approval of two major products for the treatment of nonhealing chronic wounds (topical PDGF for diabetic neuropathic foot ulcers and Apligraf for venous ulcers), it became apparent that many clinicians were either not well versed in the treatment of chronic wounds or could not easily incorporate these novel and advanced therapeutic technologies into their management strategies. The initial clues to this problem came from obviously suboptimal results when compared to those obtained in the clinical trials, or the apparent use of these technologies at inappropriate times during the healing or nonhealing trajectory of the wounds. In an editorial we published at that time [11], we laid down what we thought to be the reasons for these observations and focused on a new term, WBP, which we described as a global approach to the wound to improve its wound bed at multiple levels (optimizing the granulation tissue, decreasing the bacterial burden, removing phenotypically abnormal cells, and enhancing epidermal migration) and

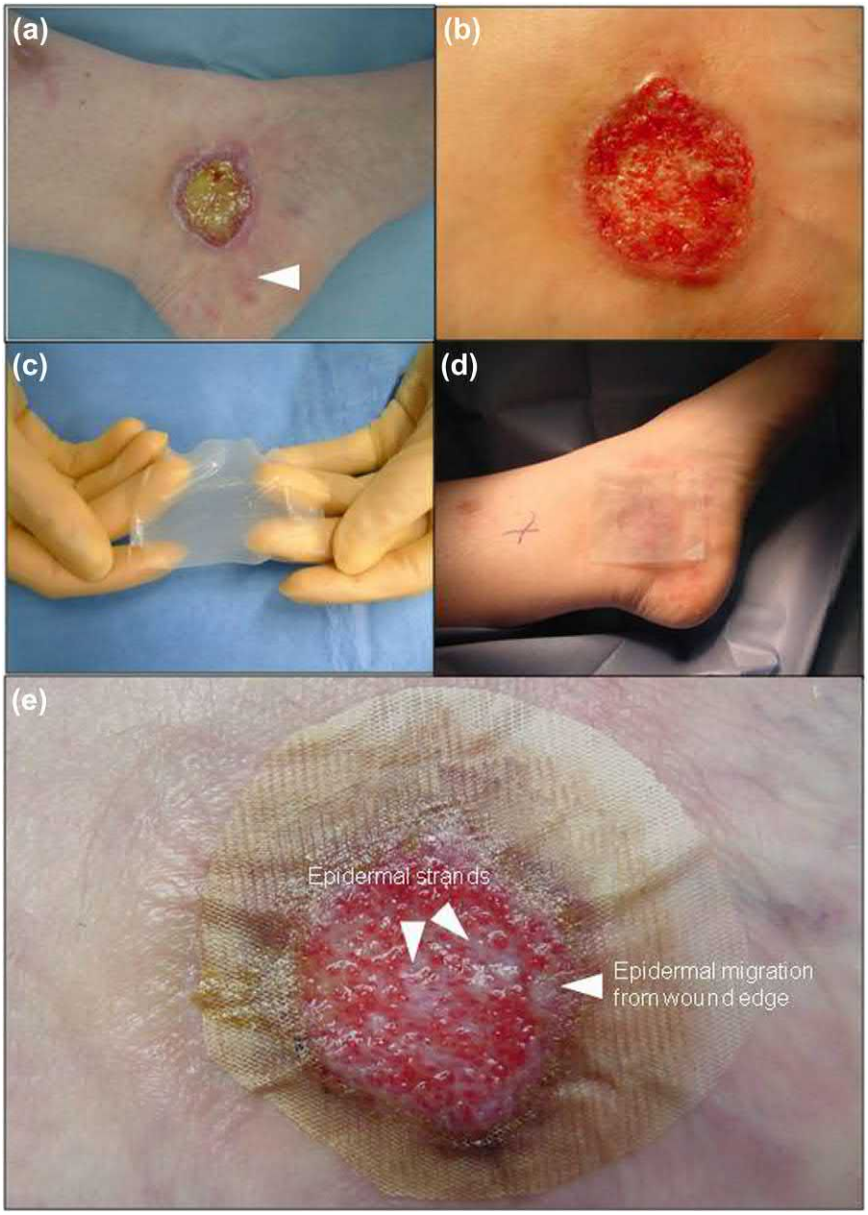

FIGURE 71.10 Treatment of vasculitis with Dermagraft.
(A) Vasculitis at baseline. Arrowhead indicates livido pattern suggestive of vasculitis. (B) Appearance of wound after surgical debridement. (C) Dermagraft prepared for application. (D) Dermagraft applied to wound. (E) Wound appearance 14 days after applying Dermagraft. Granulation tissue has developed and there is evidence of epidermal migration (*arrowheads*). *V. Falanga, ©2012.*

which would then lead to a better outcome upon treatment with growth factors, bioengineered skin constructs, and other advanced therapeutic agents. Since then, WBP has dominated the chronic wound field and has provided a more consistent way of approaching chronic wounds. Other publications on this subject have followed Refs [92,93].

There has been some struggle to define exactly what constitutes optimal WBP, but we have prepared a working scoring system to begin to investigate this issue [94]. Fig. 71.11, taken from that publication, summarizes the scoring system. The wound bed score (WBS) gives each of eight clinical parameters, assessable at the bedside a score of 0, or 1, or 2. The maximum and best score is 16. Our experience with the WBS indicates that it is a useful starting point for deciding when to use a complex and advanced biological therapy.

Proposed mechanisms of action of bioengineered skin

As stated in the previous section, there are a number of bioengineered skin products that have been designed. Still, in all cases, the exact or even main mechanisms of action (MOA) by which these bioengineered skin remain unknown. Despite this still poor understanding of mechanisms, there is some strong evidence that supports

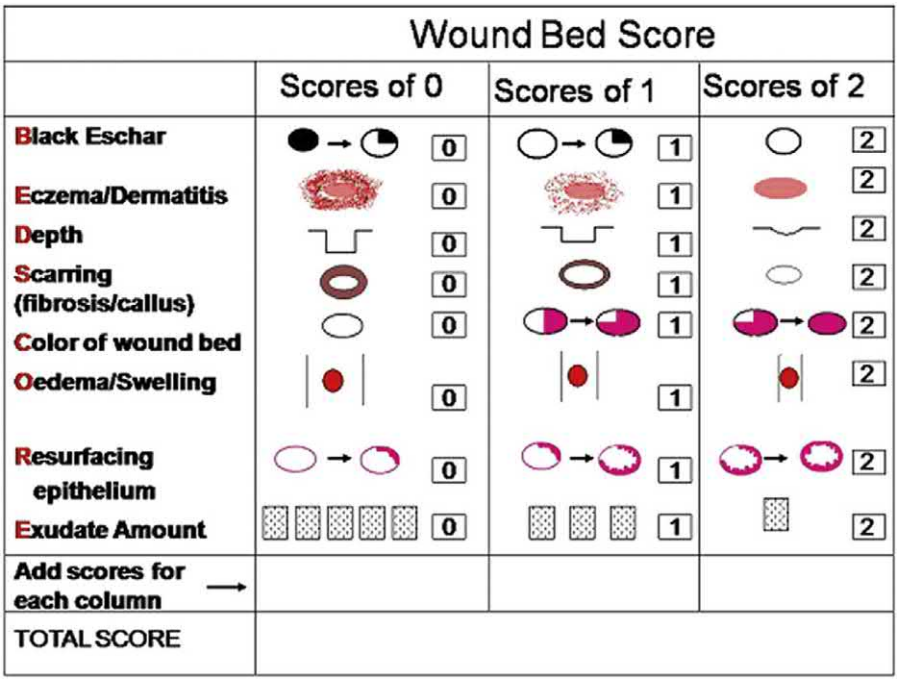

FIGURE 71.11 Wound bed scoring scheme. *V. Falanga, ©2007.*

plausible explanations of MOA. It has been stated that the delivery of living cells is associated with the release of growth factors and cytokines [62,95]. For example, it has previously been shown that, when wounded in vitro, Apligraf undergoes a staged expression of inflammatory cytokines and, later, growth factors, that is very much reminiscent of the normal process of wound healing [95]. Furthermore, a host of cytokines are produced by Apligraf, and there is evidence that its epidermal and dermal components work in concert to produce these mediators that would not normally be detected with the epidermal and dermal component alone [94].

Another interesting property of Apligraf, which is due to its unique bilayered configuration, is that it undergoes epiboly in vitro [96]. This ability to migrate over its own dermis suggests a very viable epidermal component, capable of allowing the construct to heal itself after injury [95].

The available evidence shows that the cells from several allogeneic constructs, including Apligraf, do not persist in wounds [97,98]. This has been shown in acute wounds as well as in venous ulcers, where the DNA from donor allogeneic cells is not detectable at 4–6 weeks, based on PCR-based detection of donor cells. Thus there is strong evidence that true engraftment or prolonged persistence of cells from these allogeneic constructs does not occur. Apligraf may possibly persist longer when applied to patients with certain genetic conditions, as suggested by reports that Apligraf specific DNA may be detectable many more weeks or even months when the construct is applied in the wounds of young patients with EB, a condition characterized by a variety of genetic defects in molecules responsible for anchoring the epidermis to the dermis (i.e., laminin 5 and type VII collagen) [99,100]. Still, in spite of lack of longevity of the construct, the clinical trial results indicate that even a few days or weeks of exposure of the wound to Apligraf have beneficial effects. Possible mechanisms include a more orderly and orchestrated release of cytokines, deposition of ECM material important for early migration of mesenchymal cells and keratinocytes, and even in the attraction of progenitor or stem cells from deep in the tissue or from the circulation [10].

Although it is tempting to think of Apligraf as a "graft," available evidence indicates that this construct promotes secondary intention healing in an otherwise stalled wound. We have already discussed the issue of allogeneic cell longevity, which in itself speaks against the idea of Apligraf being a graft. Moreover, there is no evidence that wound bed blood vessels grow into Apligraf, as is commonly observed with autologous grafts. Perhaps most telling is the fact that, uniformly, Apligraf seems to stimulate the edges of the wound to migrate toward the center. This "edge effect," which has also been reported with the application of living keratinocyte sheets, is the predominant observation with successful Apligraf treatment (Fig. 71.12). The edge effect strongly suggests that Apligraf does not act as a tissue replacement but, rather, stimulates secondary intention healing. Indeed, the rate at which the edges of a wound

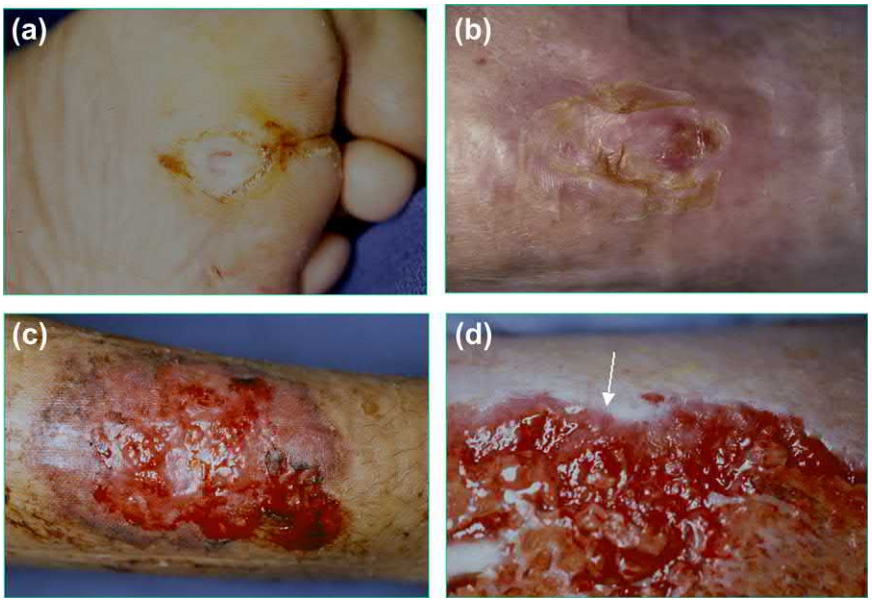

FIGURE 71.12 Different clinical appearances of wounds after Apligraf application. (A) Diabetic foot ulcer with white center representing degraded Apligraf remnants 3 week after application. (B) Venous ulcer 6 weeks after application. (C) Pyoderma gangrenosum only 2 weeks after Apligraf application and with thin layer of epidermis completely covering the wound bed. (D) Classical "edge effect" (*arrow*) at the margins of a venous ulcer treated with Apligraf. The yellow material at the bottom of the pictures represent what is left of meshed Apligraf applied 3 weeks earlier. *V. Falanga, ©2006.*

migrate toward the center has been used to determine whether eventual wound closure will occur, and Apligraf appears to accelerate wound edge migration [101]. The author has performed a clinical study, still unpublished, whereby the Apligraf is placed in chronic wounds and 1 cm away from all edges. The "edge effect" from the surrounding epidermis was still readily observed (V. Falanga, as of 2019).

Construct priming and a new didactic paradigm for constructs

Two new concepts, priming and use of the construct in conjunction with stem cells, have emerged in our understanding of how constructs could potentially be used in different ways, even without modifying the initial manufacturing process of the product. These concepts are also mentioned by one of the authors (V. Falanga) in one of his publications on bioengineered skin [101]. The priming of a bioengineered skin construct is particularly intriguing and promising, and a recent publication [102] by the laboratory team of the author shows evidence for this approach. If priming proves to be clinically feasible, it would lead to a construct containing a richer and augmented repertoire of activated genes and proteins before it is applied to the wound. It has been demonstrated that this is possible using Apligraf as proof of principle in vitro and in vivo [102]. Indeed simple incubation of the construct for 24 hours in Dulbecco's Modified Eagle Medium plus 10% FBS leads to increased transcription (up to 200-fold) of hundreds of genes related to cellular proliferation and migration [102]. The results obtained by microarray suggest that IL-6 is the most stimulated gene after using this priming step, and the results have been confirmed by western blot and by immunohistochemistry.

Another potentially useful concept, especially with the current interest in stem cells, is that bioengineered skin could be used in conjunction with stem cells to provide them with a didactic component and guidance for their differentiation. The wound bed can be treated with autologous bone marrow–derived cultured mesenchymal stem cells, and bioengineered skin can then be placed over the stem cells. We have successfully used this approach with scleroderma digital ulcers (V. Falanga, unpublished), and by delivering the stem cells with a modified fibrin spray. However, more work needs to be done to confirm these findings and to conduct appropriate clinical studies. Fig. 71.13 illustrates this type of approach [102]. The use of stem cells, including mesenchymal stem cells, can obviously considered a type of bioengineered skin. This is particularly appropriate when the stem cells are delivered topically. We showed this in a publication where autologous bone marrow–derived cultured mesenchymal stem cells were delivered in a fibrin spray to accelerate healing in murine and human wounds [103]. The polymerized fibrin in that study proved to be a rather ideal vehicle for the spraying of the stem cells onto the wound. The study made use of a double-barreled syringe, with fibrinogen in one side and thrombin in the other barrel; the stem cells were suspended in the fibrinogen solution. Upon pushing the plunger of the double-barreled syringe (connected to a CO_2 source), the cell-containing fibrinogen was mixed with thrombin, thus resulting in polymerization to a fibrin spray. The movie clip (approximately 10 seconds) shows how this approach works [104]. This initial publication has been followed by a double-blind, randomized, placebo-controlled trial in venous ulcers, which was also

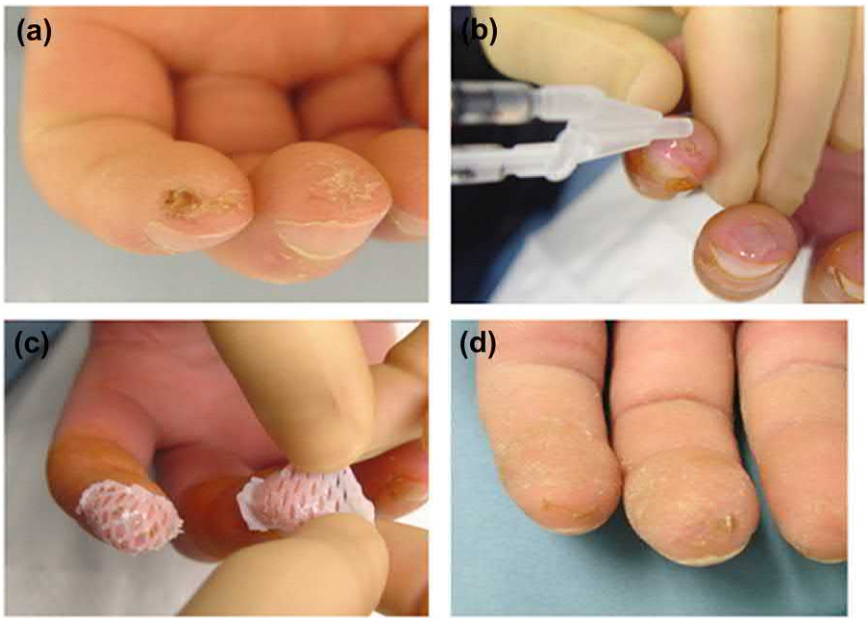

FIGURE 71.13 (A) Digital ulcers in a patient with systemic sclerosis (scleroderma). (B) autologous cultured bone marrow–derived mesenchymal stem cells being applied within a fibrin delivery system. (C) The digital ulcer was dressed with bioengineered skin (Apligraf). (D) Results observed 1 month later. *V. Falanga, ©2011.*

sponsored by the NIH. The results showed that the mesenchymal stem cells will accelerate venous ulcer healing (V. Falanga, 2019; unpublished at this time).

For every effort in bioengineered skin and its success in clinical trials of chronic wounds, there are multiple other efforts that are unsuccessful. In spite of recent regulatory guidelines that require investigators and companies to show the final results (even if not successful) there are surely situations whereby the final results are not reported in a timely fashion. One exception of the difficulties with clinical trials and bioengineered skin constructs was the study by Kirsner et al., where initially they published and reported success in venous ulcers with a staged spray of fibroblasts and keratinocytes [105]. However, this was a small study (phase 2) with a limited number of patients. Based on that initial study, a decision was made to proceed further with the smaller concentration of cells. However, when testing was expanded to a phase 3 trial, comprising a total of 673 patients with venous ulcers, no difference was found between the active group and placebo cohort [106]. The authors suggested that the negative results may have been due to differences over time in cell viability. However, it is quite possible that the phase 2 trial did not have the stringency to justify a very large and expensive phase 3 trial, at least without repeated, confirmatory testing of the cell concentration that was to be used.

Other considerations

It is very easy to be and remain excited about the prospects and the future of bioengineered skin. In the author's opinion, acute wounds and burns remain a prime target, likely leading to increasingly successful outcomes. The question is whether healing impaired wounds, where a definite healing defect plays a major role, may require a different or alternative approach. Here one needs to consider whether bioengineered skin, or similar approaches, may actually increase the energy requirement of the wound. Thus overstimulation of the wound may, possibly, not be necessarily ideal. Thus there may be an initial successful early outcome that is eventually not sustained. One possible solution is to combine the use of bioengineered skin with efforts to decrease those energy requirements of the chronic wound. This notion may require manipulation of cells, epigenetic approaches, and more creative construct materials to achieve and maintain wound coverage.

Conclusion

Bioengineered skin constructs have played a lead role in the tissue engineering field, particularly in terms of commercial successes. Like the natural tissue, these constructs are designed to mimic, the engineered tissues are proving to be dynamic in the way they respond to environmental stimuli. The understanding of how these products induce healing is evolving. Greater understanding of how engineered tissues promote wound healing is being achieved through the clinical research, presented earlier, being done with Apligraf in different wound types. Initially the action was believed to be similar to skin grafts where the patient's native tissue was replaced with the graft tissue. It is now believed that the mode of action is more complex. Stimulation of healing by secondary intention appears to be the primary mode of action; however,

there may be other mechanisms at work as well. The work being done in this area is very exciting, and researchers have only begun to scratch the surface in terms of understanding the mechanisms and environmental stimuli involved.

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Chapter 72

Principles of tissue engineering for food

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Introduction

Most techniques in tissue engineering are being developed for medical applications. The potential benefits of tissue engineering and regenerative medicine for the repair of nonregenerative organs in the human body have not really been questioned. It is generally accepted that these technologies offer therapeutic opportunities where very limited alternatives are at hand to improve the quality of life. Therefore a tremendous amount of government-funded research and business R&D has been and continues to be devoted to tissue engineering. Still, 30 years after its introduction, regenerative medicine by tissue engineering is not part of mainstream medical therapy, and developments in regenerative medicine are moving into either cell-free biomaterials [1] or into cell therapy, cytokines, or small molecules [2]. This suggests that the technical challenges to generate tissues that are fully functional and vital upon implantation are substantial.

As a spin-off from this research activity, techniques in tissue engineering and regenerative medicine may be used to produce organ tissue as food. This idea is not new and has in fact been proposed by Winston Churchill in his 1932 book "Thoughts and adventures" [3] and by Alexis Carrel [4]. Although the biological principles of tissue engineering for food are very similar to the medical application, there are also differences in goals, scale of production, cost–benefit ratio, risk–benefit ratio, ethical–psychological considerations, and regulatory requirements.

To date, no products are yet on the market, but more than 20 startup companies worldwide and one medium-sized enterprise are working toward market introduction in the coming years. They are mostly funded by private capital including investments by large traditional animal protein suppliers.

In this chapter the specifics and the challenges of tissue engineering for food production are highlighted and discussed. The focus will be mainly on tissue engineering of meat as a particularly attractive and suitable example.

Why tissue engineering of food?

Growing food through domestication of grasses, followed by other crops and by animals, had a 13,000 years head start. The success of large-scale food production likely determined the growth and sophistication of our civilization [5]. Why would we try to replace the relatively low-tech, cheap, and easy natural production of food by a high-tech complicated engineering technology that is likely to be more expensive? There are two main reasons why current ways of food production need to be reconsidered.

First, with projected growth of the world population to 9.5 billion and an even faster growth of the global economy, traditional ways of producing food, and in particular meat, may no longer suffice to feed the world [6]. Meat production through livestock, for example, already seems maximized by the occupation of 70% of arable land surface, yet the demand for meat is projected to increase by 70% over the next four decades [7]. Without change, economic theory dictates that the resulting scarcity will lead to uneven distribution and high prices. This may lead to further intensification of meat production, which will increase the pressure on crops for feeding livestock rather than people. The arable land surface could be increased but this would occur at the expense of forests with, predictably, unfavorable climate consequences. A technological alternative such as tissue engineering of meat might offer a solution. In fact, the production of meat is a good target for tissue engineering and especially beef, as cows are very inefficient in transforming vegetable proteins into edible animal proteins, with an average bioconversion rate of 15% [8]. If this bioconversion efficiency can

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be improved through tissue engineering, it will predictably lead to less land, water, and feedstock use, while the potential energy requirement is debated [9–11].

The second major reason for considering alternatives to livestock is greenhouse gas (GHG) emission. Livestock accounts for approximately 20% of the total greenhouse emission [12], partly due to fermentation of feed-in ruminants such as the cow. Fermentation produces methane that is a 20 times more powerful GHG as carbon dioxide, yet shorter lived. GHG also comes from crop growth and energy consumption across the entire chain from feed production to transport to the supermarket. Tissue engineering of meat also takes energy, but even if more energy is needed, this in the future does not necessarily equates with more GHG emission, when renewable energy will become mainstream.

In addition to these major issues with industrial dimensions of livestock breeding and feeding, there is an increasing public pressure to improve animal welfare and to reduce the risk of zoonoses.

Although there are other alternatives to avert an imminent meat and food scarcity, tissue engineering of skeletal muscle and fat to form meat is one that is being explored. It could be an excellent example of valorization of medical technologies that goes well beyond the traditional medical need and market.

Specifics of tissue engineering for medical application

The general outline of tissue engineering for food is exactly the same as for tissues constructed for medical purposes, although currently developed meat products are based on small organoids of skeletal muscle and adipose tissue or on cells only, sufficient to create minced meat variants. For larger full-thickness tissue, somatic cells are seeded onto or printed with a temporary scaffold made of biocompatible natural or synthetic polymer. Subsequently, the cell scaffold combination is cultured under tissue-specific biochemical and physical conditions to mature into the target tissue until it reaches maximum resemblance with the targeted tissue.

However, there are differences as well that could have major implications for the technologies that need to be developed and used.

Uniqueness

To avoid immunologic or physiologic host versus graft reactions, medical tissue engineering aims to create a tissue construct that is mostly personalized and produced as a unique sample or at best in limited numbers [13]. Its uniqueness is determined by its shape and form and by the cells. The shape and form is adapted to the particular environment in the recipient by, for instance, rapid prototyping, molding, or casting [14].

In many examples of medical tissue engineering, cells producing the construct are of homologous origin, thus patient derived [15]. The number of tissue-specific cells or even progenitor cells that you can harvest from patients is typically small and needs to be expanded before they can be used to populate a construct. The amount of cell culture that is required to reach sufficient cell numbers is a time- and labor-intensive process, especially when performed for a personalized, unique product. In many cases the tissue still needs to undergo some form of conditioning, which again is time and labor intensive.

Thus producing patient-specific products is labor and time intensive and therefore extremely costly. In medical applications the cost of production is weighed against the benefit for the patient, which is usually expressed in quality-adjusted life years (QALYs) that are gained through therapy. The value of a single QALY is set at approximately 40,000 USD [16], which sets the economic boundary condition of producing tissue-engineered constructs for medical application. The cost–benefit analysis is therefore very different for medical application or food production.

Function

Once implanted, the tissue-engineered products need to be functional and metabolically active, which means that they need to integrate with the circulation, the nervous system, and the endocrine regulation. The integration is not only important to sustain and coordinate function [15], but it is also crucial for homeostatic control by the recipient body. A good example is tissue-engineered myocardium to replace scar tissue in the heart that has developed after myocardial infarction. Myocardial tissue is continuously active in a highly coordinated manner and is internally as well as externally regulated. Implanted tissue-engineered constructs need to be fully integrated to maximize efficacy and minimize the risk of adverse effects (see box).

(Perhaps separate box, vignette) Myocardial tissue has an extremely high metabolism to support the arduous labor of continuously alternating contraction and relaxation. To supply the tissue with sufficient nutrients and oxygen and to remove waste, the heart is highly vascularized with a microvascular density of 1 capillary per cardiomyocyte [17]. Without sufficient blood supply, tissue-engineered myocardial constructs would die within 30 minutes. The newly implanted myocardium also needs to integrate electrically with its neighboring myocardial cells. The heart shows continuous and highly integrated electrical activity as illustrated by the typical monomorphic and regular ECG. Failure to integrate through

dyssynchronous electrical activation or through regional changes in conduction velocity can lead to lethal arrhythmias. Indeed in early trials using skeletal myoblasts to replace myocardial tissue, arrhythmias constituted the principle risk [18]. In addition to these already challenging forms of integration, the heart is a key effector in the homeostatic control of the body for instance during exercise, meals, or abrupt changes in temperature. The heart is therefore under tight control of the autonomic nervous system that regulates heart rate, contractility, and coronary flow according to requirements imposed by the body. The implanted tissue needs to respond to these stimuli in concert with the native tissue to avoid dyssynchrony in electrical and mechanical activity during exercise for instance. An even more sensitive homeostatic mechanism that is essential to coronary flow regulation is the autoregulatory control of blood vessel diameter through organ-specific myogenic responses of the vascular smooth muscle cells [19]. Organs such as the brain, heart, and kidney have an important autoregulatory blood flow control that is partly dependent on these myogenic responses. At present, it cannot be predicted how the lack of this response would affect the function of the implanted tissue-engineered myocardium, but it would be safe to optimize engineered tissues for anatomic, histologic, as well as physiologic characteristics.

The example of the heart illustrates the immense challenge for engineers and biologists to create a fully integrated tissue from cells of defined stages of differentiation and from biomaterials. Some of these challenges might be theoretical and will be resolved by remodeling of the construct after its implantation, but others might prove essential for safety and function.

Although function is a criterium in food as well, it typically refers to material (dough formation, water retention, and cooking behavior) and organoleptic functions, that is, sensory qualities during eating.

Skeletal muscle and fat tissue engineering

To understand the specific requirements of tissue engineering of consumption meat, a brief summary of the state-of-the-art techniques to tissue engineer skeletal muscle and fat is necessary. It is envisaged that the first tissue-engineered meat will be a form of processed meat such as in hamburgers or sausages, although a first proof of concept for a full-thickness meat product has been introduced recently [20]. Producing processed meat in a minced meat scenario is translated into tissue engineering of a large number of separate pieces of muscle and fat tissue that are later combined into a patty or a sausage (Fig. 72.1).

Tissue engineering of skeletal muscle

Cells

The satellite cell is considered to be the tissue-specific stem cell, responsible for in situ regeneration of damaged muscle, although more pluripotent skeletal muscle–derived stem cells have been described as well (Figs. 72.1 and 72.2) [21]. Culturing of skeletal muscle cells from satellite cells includes a proliferation phase of myoblast offspring followed by further differentiation into skeletal muscle.

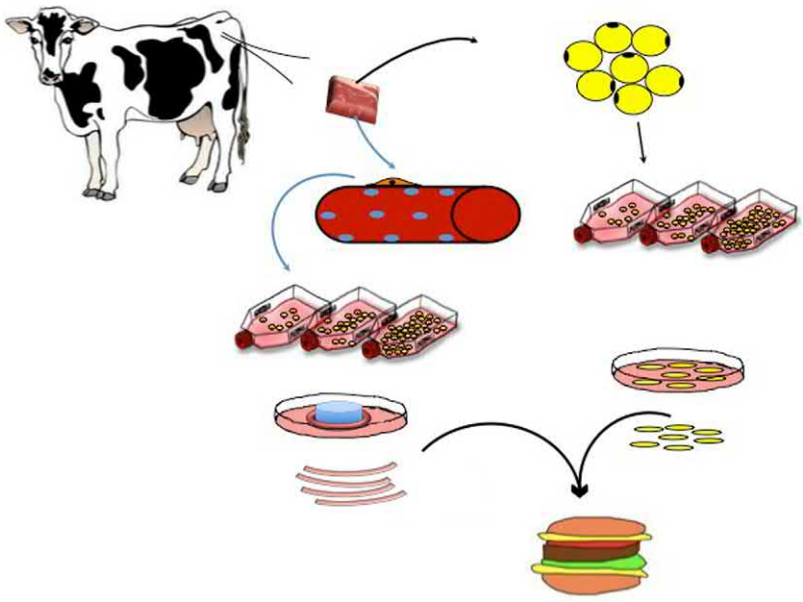

FIGURE 72.1 Tissue engineering of meat.
From a biopsy of a cow, ASCs and satellite cells are harvested and cultured separately. The satellite cells are seeded in a gel onto a culture plate in a ring around a central column. They self-organize into a bioartificial muscle. The ASCs are differentiated into adipocytes and cultured in a gel or scaffold, without the need for anchors. Minced meat, a hamburger, for example, will be constructed from a mixture of bioartificial muscles and fat tissue. *ASCs*, Adipose tissue–derived stem cells.

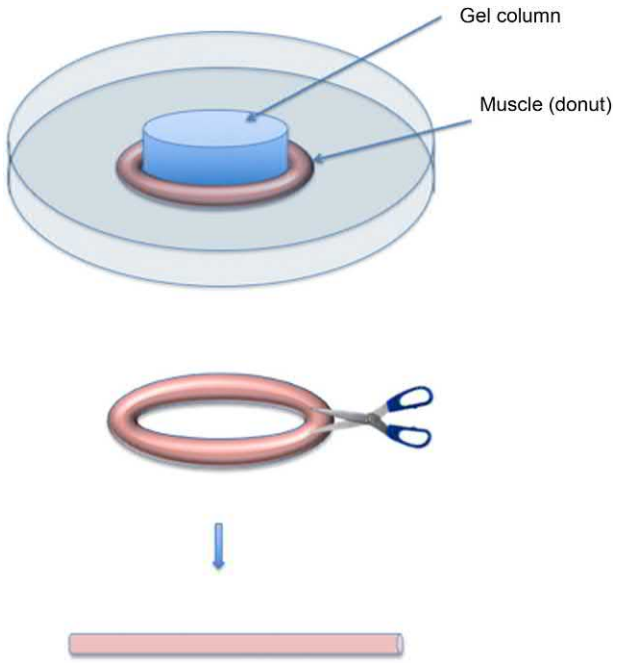

FIGURE 72.2 A bioartificial muscle from bovine satellite cells, 3 weeks after loading the gel onto the culture dish in ring structure around a central column. After 3 weeks the rings are harvested, cut open and as strands incorporated into meat product.

The goal of the proliferation phase is to maximize the number of doublings. With the current isolation and culturing methods for myoblasts, more than 30 doublings can be achieved. With proper conditions, this can probably be improved to 50–70 doublings [22], in accordance with the Hayflick limit. A major improvement to keep the myoblasts in the replication phase comes from harvesting them through a combination of mild enzymatic treatment and trituration of skeletal muscle fibrils according to Collins et al. [23], although the exact optimized method may be species specific. During harvest the stem cell behavior is maintained in a niche environment [24]. The elasticity of the substrate, for instance, is important to maintain the stemness of satellite cells that are cultured [25]. During the proliferation phase of myoblasts in our hands, the effect of physiologic substrate stiffness on proliferation was mild, higher than on very flaccid substrates (3 kPa range) but not significantly different from stiff, plastic, and surfaces [26]. Coating the culture surface with laminin and collagen IV, two major basal membrane proteins, has also some impact on the proliferation rate of myoblasts [27]. In addition, there are many biochemical stimuli reported to have an effect on stemness such as TGFβ1, Pax7, Notch, and Wnt [28], which should lead to higher doubling numbers. These mechanisms can be targeted with specifically designed agonists or with known biological modulators in order to delay differentiation. For instance, we recently established that inhibition of MAPK P38 prolongs stemness and increases the amount of doublings in bovine myoblasts without affecting their differentiation capacity [29]. Once sufficient numbers of cells have been generated, groups of cells can be stimulated to differentiate.

Gel and seeding

Differentiation is primarily initiated by reducing the serum concentration in the medium. Cells are typically cast in a collagen gel with or without Matrigel supplement or they are seeded into a biodegradable polymer structure. When taken up in the gel, it is important to provide anchor points in the culture dish. Alternatively, the cell–gel mixture can be deposited around a central column. Typical for mesenchymal cells, differentiating satellite cells will organize the gel to the smallest structure possible. For this the gel has to be functional so that cells can contract the gel.

In addition to the specific functionality of the gel, the conditions for the biomaterial component of muscle (and fat) tissue–engineered constructs for food purposes are as follows:

1. Nontoxicity of biomaterial or its degradation products upon digestion.
2. Nonallergenicity of biomaterial or its degradation products upon digestion.
3. Residual biomaterial should be digestible.
4. Supply of material in industrial quantities, not coming from livestock.
5. Material should be cheap.

Any of these characteristics is unchartered territory and the present information on biomaterial–cell combinations should be reevaluated in the light of these requirements.

It is likely that nontoxicity of ingested material correlates with biocompatibility in current cell-based assays. If a particular biomaterial is not cytotoxic in direct exposure to cells, it probably is not toxic after ingestion either. In fact, the absence of toxicity in cell-based assays might be too stringent a criterium as some materials will be digested before absorption or may not be digested or absorbed at all. These reasonable hypotheses, however, should all be formally tested for each biomaterial as it is equally conceivable that rapid mechanic and enzymatic degradation by the gastrointestinal system release other metabolites that are cytotoxic. The biomaterials that have been tested with muscle cells and adipocytes all comply with the requirement of being noncytotoxic, but none of them have been tested after oral intake.

Nonallergenicity is less obvious. Most ingested proteins or materials do not lead to an allergic reaction, but some foods, such as eggs, milk, shellfish, and peanuts,

are particularly allergenic [30]. Biomaterials used in tissue engineering for food therefore will have to be tested for allergenicity as well.

In a functionalized gel, satellite cells or myoblasts will gradually compact the gel in a process called gel contraction or collagen contraction if the gel is collagen based. With the anchors provided the smallest configuration upon compaction is a thin tissue strip in between the anchors. When the cell−gel combination is placed in a ring, it remains a ring structure, just more compacted. This will later form the myooid or bioartificial muscle (BAM) (Fig. 72.2) [31]. Differentiation leads to the merging of myoblasts into primitive skeletal muscle cells, called myotubes. At this time, the cells will start to express early stage skeletal muscle markers such as MyoD, myogenin, and embryonic isoforms of muscle myosin heavy chain [32]. This cues for subsequent hypertrophy: metabolic, biochemical, and mechanical. After organization into a BAM and with subsequent differentiation, the muscle will develop increasing tension in the ring structure as it compresses the noncompressible central column. The resulting tension is a major trigger for protein production [33], the optimization of which is the final goal of tissue engineering of food. Several other mechanical stimuli such as passive cyclic stretch and electrical stimulation have been investigated. Interestingly, cyclic stretch did not further improve protein synthesis but had in fact a slight negative effect [34,35]. This result is somewhat controversial as others have observed positive effects of cyclic stretch on muscle maturation [36]. In addition to passive stretch and tension, we and others have investigated the effect of electrical stimulation to further stimulate protein production and force generation [37]. In combination with specific coatings, electrical stimulation did lead to earlier maturation of the skeletal muscle fibers and more protein production. More extensive data on the modes and efficacy of mechanical stimulation for muscle differentiation can be found at Refs. [38−40].

With the above-described techniques, it is feasible to generate BAMs of small dimensions, with limited mass transfer of nutrient and oxygen through diffusion. No attempts have been made yet to create large BAMs with a built-in blood vessel or channel system conducting a continuous flow of oxygenized, nutrient-rich medium. However, printing and biomaterial technologies have been described, which would make this possible and certainly testable [41−43].

Although contractile proteins comprise the bulk of protein content and quality of muscle tissue, there are other proteins that are important for texture, color, and taste of the muscle tissue. One particularly important protein is myoglobin. As a heme-carrying protein it is responsible for the pink color of meat and a major carrier of iron. It contributes nutritional value and will likely determine taste as well. The transcriptional regulation of myoglobin is reasonably well understood and involves the transcriptional activators MEF2 and NFAT/calcineurin [44] and coactivator PGC-1α. It appears that contractile activation of muscle in the setting of hypoxia will stimulate myoglobin maximally. We have indeed observed that in differentiating bovine myoblasts the expression increases with decreasing oxygen concentration (unpublished data). It is therefore feasible to increase the myoglobin content using stimuli that are compatible with tissue engineering for food.

In summary the effective culture of skeletal muscle is possible with current technology. There are numerous options for refinement and extension suggesting that it will take time and effort to optimize the product. In 2013 we presented the first hamburger made from cultured bovine skeletal muscle that was cooked and eaten (Fig. 72.3). It was identified as meat by the tasters and was reported to have a meat-like texture. It was presented as a proof of principle and not as a real product yet as exemplified by the astronomical cost and the fact that a traditional, nonscalable, cell culture method was used for its production.

Tissue engineering of fat

Cells

Tissue engineering of fat tissue is less advanced than that of skeletal muscle because the medical need seems less pressing. Indeed, for the purpose of plastic surgery, tissue engineering of fat might be applicable in the clinic and scant efforts have therefore been made to create adipose tissue constructs [45]. However, differentiation of mesenchymal stem cells (MSCs) into adipocytes is one of the hallmarks of MSC phenotyping, and as a result the

FIGURE 72.3 The World's first hamburger cultured exclusively from bovine myoblasts. The hamburger was presented to the public in August 2013. The individual strands are clearly identified.

various steps toward differentiation into adipocytes have been described in great detail.

Preadipocytes are precursors of fat cells and characterized by the expression of PREF-1 (preadipocyte factor 1 aka Dlk-1 or delta-like 1 homolog), CD105 (endoglin), and the absence of CD45 and CD31. These cells are typically fat tissue derived and therefore often referred to as adipose tissue−derived stem cells (ASCs). ASCs are extracted using a combination of mechanical disruption and collagenase treatment. ASCs adhere to plastic and form initially a heterogeneous population [46] that can differentiate into adipocytes, chondrocytes, and osteocytes. Unfortunately, these cells undergo limited proliferation, and after a couple of passages, they lose their capacity to differentiate [47] and are therefore not suitable for tissue engineering for food. In contrast, human multipotent adipose-derived stem cells (MADS) that are derived from infants continue to proliferate and retain their differentiation capacity [47]. It remains to be shown whether this is a consistent finding across species. If so, MADS can be harvested from young animals, and these cells can then be used for tissue engineering of edible fat. An alternative cell source for the production of fat tissue has been obtained by so-called ceiling culture of digested human adipose tissue [48]. In this technique, two types of fibroblast-like cells can be distinguished, both of which have a strong capacity to proliferate while retaining their ability to differentiate into adipocytes. It is likely that these cells can be harvested from bovine adipose tissue, as it has already been successfully done in humans, rats, and sheep [48].

Finally, it is possible that satellites themselves may differentiate into adipocytes as shown by Asakura et al. in mouse satellite cell−derived primary myoblasts [49]. The species dependency of this phenomenon requires further study.

Biochemistry of adipocyte differentiation

Differentiation of preadipocytes is typically initiated by starvation, that is, reduction of fetal bovine serum (FBS) and a temporary cocktail of isobutylmethylxanthine (IBMX), dexamethasone, and insulin. In some protocols, triiodothyronine [50] and/or the PPAR-γ ligand rosiglitazone [51] are added to the differentiation stimulus. It remains to be shown how much and if any of these stimulators are still present upon harvesting of the tissue after 3−4 weeks of differentiation, but it is safe to assume that these agents should be replaced by approved food additives (see next). IBMX is a very potent methylxanthine and a nonselective phosphodiesterase inhibitor that is not approved as a drug. IBMX induces the activation of PPAR-γ, a critical transcription factor required for adipocyte differentiation [52]. It probably acts through induction of intracellular cAMP. If so, other agents that act through induction of cAMP and that are approved for drug or food purposes could replace IBMX. Dexamethasone is a glucocorticosteroid that acts through the intracellular glucocorticosteroid receptor (GR) with a number of intermediate products that eventually upregulate C/EBPα (CCAAT enhancer binding protein). Together with PPAR-γ, C/EBPα is a key transcription factor for adipogenesis. The steroid-stimulated GR requires interaction with promoter elements in the intermediate products; therefore it will be difficult to find a suitable substitute. The third component is insulin, which is typically short lived and natural, and is therefore less likely to be a regulatory issue.

Given that most free fatty acids (FFAs) are natural ligands for PPAR-γ, we and others set out to study a range of saturated, ω-5, ω-7, and ω-9; unsaturated; and branched FFAs for their ability to differentiate bovine preadipocytes and indeed found some FFAs that have the same performance as the dexamethasone/IBMX/insulin cocktail while being perfectly food compatible [53].

Scaffold and cell seeding

The fat cells can and should be captured in a scaffold to form fat tissue. The same considerations for the biomaterial apply that were presented for skeletal muscle tissue engineering, yet the specific biochemical and physical cues needed to mature and maintain fat cells are different from satellite cells. It is evident from the experience of many investigators that mature fat cells are fragile, prone to apoptosis, and are terminally differentiated. For that reason, most cell seeding on scaffolds is performed in the preadipocyte stage [54−57] or is even performed with cell lines such as the mouse 3T3L1. In the vast majority of studies, the constructs were implanted at an early stage, and in vivo adipogenesis was allowed to develop for a couple of weeks after which the state of differentiation was assessed. This suggests that it is difficult or time-consuming to reach full adipocyte differentiation and mature fat tissue by in vitro conditioning alone. A good summary of material and cell combinations that have been studied can be found at Refs. [58,59].

Bio-based materials that have been used for engineering adipose tissue include collagen [60], hyaluronic acid [61], fibrin [62], and chitosan [63], which are already present in our diet; so it is not to be expected that tissue-engineered food with these materials will augment the risk of toxicity or allergy.

Of the synthetic or semisynthetic biomaterials, polylactic-co-glycolic acid (PLGA) [64] and esterified hyaluronic acid [61,65] are the most promising in terms of in vivo differentiation of adipocytes. In some studies the addition of FGF-2 seemed to facilitate adipose

differentiation. Confirming the results of others, but using bovine ASCs, we observed little development of fat tissue in electrospun PLGA scaffolds and fibrin sheets, but acceptable adipogenesis in alginate and in RGD functionalized alginate (unpublished data). A recent study showed full-blown human adipocyte differentiation using a combination of alginate and collagen [66], strongly suggesting that the right choice of biomaterial can be crucial in driving adipogenesis.

Similar to skeletal muscle engineering and MSC differentiation, stiffness of the matrix may be an interesting parameter to affect specification and maturation of ASCs in vitro [67]. Human bone marrow–derived MSCs assume quiescence when placed on a 250 Pa surface, which represents the natural elasticity of fat tissue. In this quiescent state, they cease to proliferate, enter differentiation, and develop into adipocytes that form adipose tissue [68].

As of late, the use of decellularized extracellular matrix has been gaining attention in regenerative medicine [69]. It is obvious that this technology will not be applicable in tissue engineering of food since it requires harvesting massive amounts of original tissues, and the animals would still have to be bred as donors of these tissues.

In summary, growing edible pieces of fat through tissue engineering requires an optimal combination of biomaterial and chemical, biochemical, and physical conditioning. The goals are in part different from fat tissue engineering in, for instance, reconstructive surgery, and many variables have yet to be optimized.

Specifics of food tissue engineering

The two major advantages of tissue engineering for food are that the eventual product can be made in large series and that it requires no functional integration. Instead, food tissue engineering faces at least three new challenges.

The first is the need to generate sufficient tissue volume to feed large populations. The scale of cell and tissue culture will be several orders of magnitude higher than needed for medical applications: the hamburger presented in 2013 was made from 10^{10} bovine myoblasts. The focus of tissue engineering for food will therefore shift from creating individualized constructs to mass production. This shift has major implications for bioreactor design, biomaterial selection and production, culture medium optimization, optimization of tissue conditioning, and quality control of, for instance, the genetic stability of the cells.

Second, food production by tissue engineering can only be a viable alternative to conventional livestock meat production if the bioconversion of vegetable proteins to edible animal proteins is more efficient than in the common domestic animals such as cows and pigs. To attain this efficiency in cell culture, it is highly likely that medium has to be recycled after having been spent.

The third challenge is that tissue-engineered food needs to be very similar to, if not exactly the same as, the conventionally produced food it intends to replace. Meat, for example, has been replaced with very limited success by products made of vegetable proteins. The typical texture, color, and taste of meat are apparently still very difficult to mimic through current innovations in food technology, although the recent addition of leghemoglobin to plant-based substrates gives the appearance of "bleeding" and arguably the required taste. In some cases the science of these characteristics is not far enough advanced to design rational strategies for synthesis. For instance, the chemistry of the taste of meat is very complex and incompletely understood and therefore mainly relies on subjective analysis by taste panels [70]. The only rational strategy therefore is to strive for the best mimicking biochemical composition, that is, the one most closely resembling that of livestock meat.

Scale

The volume of global meat production is 330 million tons/year of which 79.8 million tons is beef [71]. Given that each gram of muscle tissue contains approximately 5×10^6 cells, this amounts to 1.5×10^{21} cells/year, provided that all muscle progenitor cells differentiate into mature skeletal muscle cells and get incorporated into skeletal muscle. This number may be an order of magnitude off, due to inaccuracies with respect to muscle type, fat content, etc., but it provides the rough scale to which cell production needs to be industrialized.

Currently, large bioreactors contain up to 25,000 L or more with a maximum cell load of 7×10^6 cells/mL so 35×10^9 cells/bioreactor [72,73], provided that the cells are cultured in suspension. Mesenchymal cells such as satellite cells or myoblasts need to adhere to surfaces to sustain proliferation and survival. Several microcarriers have been designed to grow large numbers of adherent cells in bioreactors [74]. This would require a stage in the process where cells are again detached from the carriers, which could be as simple as just changing temperature [75]. The limits on bioreactor size and number of cells per mL further depend on a number of factors such as oxygen transfer, nutrient delivery, and washout of waste. These factors are all related to unequal mixing of the fluid. Stirring or otherwise agitating the cell mixture might solve most of the problems, but there is a limitation to the agitation that mammalian cells can handle in the absence of a firm cell wall that withstands high shear stresses [76].

As a result, the cells may suffer from insufficient and inhomogeneous transfer of oxygen and nutrients [76].

Inadequate mixing of bulk fluids also becomes more difficult as the volume of the bioreactors increases. Simulations in smaller tanks show that gradients of pH and nutrients reduce cell growth and maturation [77]. Another important factor in scaling-up cell culture in large bioreactors is adequate removal of CO_2. Carbon dioxide is typically removed from the medium through a combination of agitation and sparging: controlled air bubble inlet to retrieve CO_2 from liquid and bring it into the gas phase. Accumulation of CO_2 presents an important limit to mammalian cell culture due to the relatively low agitation and air sparging rates that are utilized [78]. The optimal dissolved carbon dioxide (dCO_2) level at which cells thrive varies between cell types, but relatively high dCO_2 levels universally inhibit cell growth and protein production [79,80].

Even when the technical limitations of large-scale cell culture are overcome, the sheer number of cells needed to produce sufficient meat to feed the world population in 2050 would still require a mind-boggling amount of bioreactor space. Assuming that each bioreactor has a 4-week turnover of its cell content and would therefore have 13 operation cycles per year, one would still need 600 million bioreactors of 25,000 L, roughly 1 for every 10 humans, again the numbers can be an order of magnitude off. These already staggering figures do not even take into account that a seed train is needed to get sufficient cells for the initial start of a large volume bioreactor culture [73]. In addition, for skeletal muscle production to produce protein-rich and firm muscle tissue, the proliferation phase should be followed by differentiation and conditioning of the muscle progenitor cells, which also requires bioreactor space.

For this volume of bioreactor space, one needs equivalent volumes of culture medium. The production and storage of these amounts of medium will be a logistic challenge unless powder formulations are being developed that can be easily reconstituted within the same production line as the cell culture. In making powder formulations of medium, the addition sequence and the milling equipment are important parameters [73].

Directly related to the scale of cell culture is the quality control of the progenitor cells. Cells may undergo differentiation toward undesirable phenotypes, they may be epigenetically modified or they can accumulate karyotypic abnormalities over time. For safety and efficacy reasons, these undesirable outcomes should be detected and controlled [81,82]. Current technologies such as PCR and fluorescent in situ hybridization are not designed for large sample sizes or for in-line monitoring of quality. Several other techniques based on surrogate physical parameters such as electric conductivity or Raman spectra have been developed, which are capable of in-line monitoring of the cell culture process, which will hopefully be sufficiently robust to guarantee the quality of the cell culture [83]

From these considerations, it may be clear that scaling-up mammalian cell culture to the level that is needed for safe and high-quality production of one of our basic foods is a nontrivial enterprise and involves substantial innovation through chemical and mechanical engineering in close collaboration with cell biologists.

Efficiency

Although cell culture of mammalian cells has been evolving over the last five decades, relatively little attention has been paid to optimizing the culture process in terms of efficiency.

In such an optimization strategy, hundreds of variables can be—and have to be—controlled to make the process reliable and efficient. These variables include all individual components of medium and serum but also physical culture conditions. The vast number of variables that likely interact and therefore need to be approached in their contexts creates challenges as well as opportunities. The level of each variable (e.g., feed component, biochemical, and biophysical culture conditions) and the possible interactions with other variables need to be established. Current culture protocols have largely been developed through trial and error, leading to a gradual optimization, whereas it clearly requires a more systematic approach. For bacteria and simple eukaryotic organisms such as yeast, a systems biology approach is starting to be developed [84–86]. Ideally, a biological systems strategy should be established for more complex mammalian cells as well. Alternatively, a large-scale, high-throughput analysis is used to optimize culture media. This is true for the nutrient part of the culture medium as well as for the FBS, together, a formidable task. Cell culture media for tissue engineering of food and arguably for other applications as well should be completely synthetic and chemically defined. A limited number of such products have been developed for medical purposes, and it is to be expected that more of these will become available [87]. In our hands, a selection of xeno-free media supports bovine myoblast proliferation, although serum-based media is still superior (unpublished data).

Opportunities to increase the efficiency of skeletal muscle cell culture are also numerous. In the production phase, recycling mechanisms and combining culture with nutrient supplying systems through, for instance, photosynthesis would create substantial benefit and value [11,88]. Given the scale of cell culture, these recycling mechanisms need to be an integral part of the production chain.

Taste, texture, juiciness

A large number of small peptides and fat dissolved aromatic substances have been described that contribute to

the taste of meat [70,89–92]. However, the relative importance of each component is unknown. The reaction between certain sugars or glycosylated peptides and fatty acids probably interacts especially during cooking of the tissue to create the specific taste of meat in the so-called Maillard reaction [93,94]. Taste is a subjective sensation and blinded panels in fact still primarily judge the taste of meat. To add to the complexity, in addition to the chemistry of the meat, the cultural background of the panelists appears to weigh in on the evaluation of meat products [70]. It has become clear that the taste of meat can be affected by feed and not so much by the strain of cattle [91], suggesting that the feed conditions of the cultured skeletal muscle can also be important for taste development. Even postmortem conditions determine the taste of meat as a result of oxidation of proteins, sugars, and fatty acids during controlled decay of the muscle [90]. Thus in tissue engineering of meat, taste is an extremely important feature to be investigated and taken into account. On the other hand, it should be borne in mind that today most processed meats are artificially flavored. The flavor industry has reached a level of sophistication where numerous flavors can be added to food components with such subtlety that it stays unnoticed by the nonexpert consumer.

Texture of meat is mainly determined by the presence of intramuscular connective tissue and its perimysial distribution [95] and the amount and distribution of fat [96]. Fat is also the main determinant of juiciness, although the percentage of fat in various meats shows modest variability between 0.5% and 8.0% with a 3% average [97]. As intramuscular connective tissue and content can be affected by feed and cell mixture, these characteristics can likely also be optimized in cell/tissue culture and should be primary targets to produce high-quality meat.

It is not obvious that tissue engineering for food is a cheaper proposition than other alternatives such as vegetable protein meat replacers. Therefore the outcome should be indistinguishable from livestock grown meat in terms of taste, color, texture, and juiciness. The quality should also be constant and preferably more predictable than for the natural product.

Although primarily meant as a credible alternative for consumption meat, culturing of meat under precisely determined conditions will greatly contribute to meat science in general and in particular to the determinants of taste, texture, nutritional value.

Enhanced meat

Tissue engineering of meat enables the creation of novel products. All vertebrate animals have satellite cells in their muscles, and currently, there is reason to believe that skeletal muscle can be tissue engineered from these species. We have found that very little adjustments in protocols were needed to transfer satellite cells from mice, rats, pigs, or cows into mature skeletal muscle. Novel meats from hitherto unused species or from a combination of cell sources could be made. It is, however, more important to design and produce cultured meat that is healthier than the original product.

One particularly interesting example is to increase the content of conjugated linoleic acid (CLA) (octadecadienoic acid 18:2). CLA is naturally present in meat, and the main source of our dietary CLA is beef fat and bovine milk [98]. CLA is formed in the stomach by bacteria that conjugate linoleic acid, which is subsequently taken up by the fat cells. This mechanism can be used to load tissue-engineered fat with CLA to increase it to levels that are associated with anticarcinogenic, antidiabetic, and antiatherogenic effects, as well as a strengthening of the immune system, bone metabolism, and improvements in body composition [98].

Another health issue associated with the consumption of red meat is the increased risk of colorectal cancer [99]. It is not completely clear what the causal factor in meat drives the increased risk, but heme iron or its associated oxidative effect has been frequently incriminated. If this indeed turns out to be a causative factor, meats can be produced with less heme iron content, since this content is already lower than normal when culturing under ambient oxygen conditions.

Other foods

Although tissue engineering of meat is probably the first and best example of food production through this technology, there are other foods that could be tissue engineered. Currently, the pressing need to create an efficient and environmentally sound alternative to agricultural food production focuses our tissue engineering endeavors first and foremost on beef. Other staple meat products are being pursued for different reasons such as poultry to improve animal welfare, or fish, for maintenance of biodiversity. In fact, the first showcase of tissue-engineered food constituted goldfish meat to be used by astronauts on their space missions [100].

Milk production by tissue-engineered mammary glands might also be feasible in the future. If cows are no longer needed in large numbers for meat production, an alternative method to produce milk should also be found. Bovine mammary gland stem cells can be isolated with techniques described earlier for their human counterparts. The bovine stem cells recapitulated the organization of bovine mammary tissue, and they produced milk. The investigators conclude that there is a potential for novel engineering and transplant strategies and a variety of commercial applications, including the production of

modified milk components for human consumption [101]. Alternatively, an arguably easier, milk is reconstituted from, among many other components, proteins such as lactoglobulin, whey and casein that are made through recombinant technology [102].

Last, organ meats such as stomach, liver (foie gras), kidney, or sweet bread might be future target foods to be tissue engineered.

Consumer acceptance

Cultured meat cannot be bought yet; how to produce it is still the subject of increasingly intensive research and development. At the same time the last decade has seen many attempts to estimate as precisely as possible how promising it is as a potential solution for the problems of traditional meat. For example, the first—very tentative and very promising—life cycle analysis (LCA) dates from 2011 [11] and several more have followed [9,10]. More recent LCAs no longer see energy reduction as a very promising aspect of cultured meat, while other advantages, such as great reductions in land use, still hold. Such attempts are informative but also have inherent limitations, since companies are still working hard on production processes, including their efficiency and sustainability. Similar limitations apply to attempts to predict consumer acceptance. For many people, cultured meat is still an unexpected idea that they find very strange when they first hear of it. Just like the technological development of cultured meat, its societal development is a process that takes time. In the course of this process the idea slowly becomes more familiar, its meaning is being explored from various sides, and mixed feelings are sorted out. Measuring responses at any particular moment cannot do justice to such time aspects and, perhaps not surprisingly, results of consumer surveys differ greatly, depending, for example, on how questions are framed [103]. In order to understand changes in the run-up to the introduction of cultured meat, getting in touch with the relevant processes, complicated though that may be, is needed.

Meat is in motion. For decades now the problems of traditional meat have been increasing, and its moral reputation is in decline. Mixed feelings are all around: while most people find meat delicious and consider it a cherished part of their traditional diet, many also increasingly worry about animal suffering, the ecological footprint of meat eating, and the health aspects of eating too much of it. Announcements and expectations of cultured meat have become part of this moving field, together with other alternatives. We aimed to get in touch with this process of change through studying responses to cultured meat over the years, complemented with workshops and focus groups. Focus groups allow people at least a little time, following their first surprise, to consider and discuss the issue.

In focus groups the idea of cultured meat always quickly triggered mixed feelings about both cultured and traditional meat. For example, when someone commented on the unnaturalness of cultured meat, someone else was bound to wonder how natural our ordinary meat still is, and discussions then shifted to the many ambivalent feelings concerning meat. This led to changes in meaning and appreciation: after such discussions, normal meat had become somewhat less normal, cultured meat had become somewhat less strange. This shift was also illustrated in the *Times* the day after the proof-of-principle hamburger of 2013, when the commentator wondered "How absurd is it to imagine all our meat one day being produced by a similar [tissue culturing] process? Not much more absurd than it is to imagine all our meat continuing to be produced as it is now" [104].

Ambivalence is psychologically uncomfortable. One way to diminish such discomfort is to change behavior, but other forms of "coping behavior" are also available, such as "strategic ignorance": not wanting to know too much, in order to avoid the discomfort. In a study on food and information, we indeed found that many people avoid information about meat, not because they are indifferent but in order to cope with ambivalence [105].

Although many people may not be quick in changing their meat consumption, they do gradually become more open to change. In our focus groups, age was a factor here. While people of all ages after some discussion tended to sympathize with the idea of cultured meat to some extent, older people were more explicitly open to the prospect of changing diets, based on their more explicit mixed feelings toward traditional meat and also on their experiences of earlier diet changes. Margarine was also very strange at first, as the oldest among them remarked. They contemplated on how they were not immediately inclined to adapt individually ("I like meat far too much"), but instead hoped for change on other, more collective levels ("McDonalds should do this") [106].

Will big companies take over the role of farmers? Cultured meat will be disruptive for traditional livestock farming; it is clearly a threat for farmers. But it has also been proposed that cultured meat might perhaps be best produced on a small scale, even on farms, in reactors fed by cells from free-ranging animals—so that it might in fact become a new opportunity for farmers [107]. This is part of the search for how cultured meat might become a societally attractive option.

Societal indecisiveness and a lack of clear direction may be typical of (early) periods of big moral change: many people are not ready for individual action, but "under the surface" the status quo becomes instable.

For quite a while, individuals and groups remain busy sorting out the issues: finding their traditional distinctions and frameworks no longer quite adequate, making sense of new options, fitting in with technological and social environments in new ways. Understanding these processes requires us to pay attention to ambiguous meanings and ambivalent evaluations; they may look formless but they involve important moral work. The imminent appearance of cultured meat products on the market will no doubt lead to more concrete directions in this moving field.

Regulatory pathway

Because of the manufactured nature of cultured meat, it will likely have to undergo specific regulatory approval before it can enter the market, equivalent but not equal to new medical therapies or devices. In Europe, cultured meat has been designated a "Novel Food," which means that the European Food Safety Agency (EFSA) has to approve cultured meat as being safe under the just-revised Novel Food regulation [108]. Once EFSA has approved cultured meat, member states are likely to open their markets to cultured meat. Postmarket surveillance is then executed at a member state level. A similar process may be followed in the United States, where FDA and USDA have stated to jointly regulate cultured meat. This process will take time and effort, but when food safety considerations guide the choice of processes and materials during the production of cultured meat, there is no reason to assume that there will be safety issues.

Conclusion

Cultured meat is an interesting and potentially very useful application of tissue engineering. It shares technologies with medical applications, but in the implementation, there are major differences not only in scale, economy, social acceptance, and regulation but also in functional requirements. Taste, texture, and cooking behavior are important functions and are not easy to achieve. On the other hand, there is no need for structures that require functional integration of the tissue upon implant, thus reducing complexity. Overcoming the specific challenges of tissue engineering for food may have spillover effects to tissue engineering for medical therapy, for instance, on large-scale cell production.

Tissue engineering for food is still in its infancy as there are currently no products on the market yet. However, momentum is increasing as exemplified by the rapidly increasing business activity. At the same time, rigorous scientific work is needed in order to reach the full potential of cultured foods such as meat.

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Chapter 73

Cultured meat—a humane meat production system

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Introduction

Current meat production systems are a major source of pollution and a significant consumer of fossil fuels, land, and water resources. Nutrition-related diseases, foodborne illnesses, resource use and pollution, and use of farm animals are some of the serious consequences associated with them. Globally 30% of the land surface is used for livestock production with 33% of arable land being used for growing livestock feed crops and 26% being used for grazing [1]. The livestock sector contributes 18% of anthropogenic greenhouse gas (GHG) emissions to the atmosphere worldwide [1]. This contribution, as measured in carbon dioxide equivalents, is higher than the share of GHG emissions from transportation [2]. The livestock sector produces 37% of anthropogenic methane, which has 23 times more global warming potential than carbon dioxide, and 65% of human-related nitrous oxide, another powerful GHG that has 296 times more global warming potential than carbon dioxide, most of which comes from manure [2]. Livestock also contributes 68% to total ammonia emissions [2]. The animals themselves are mostly responsible for the emission of GHGs [3]. The demand for meat is expected to grow worldwide, and global meat production is anticipated to increase to 465 million tonnes by the year 2050, which is anticipated to be accompanied by a rise in annual GHG emissions to 19.7 Gt of carbon dioxide, carbon equivalent [1]. Meat production is also responsible for the emissions of nitrogen and phosphorus, pesticide contamination of water, heavy metal contamination of soil, and acid rain from ammonia emissions [4]. The water used for livestock and accompanying feed crop production has a dramatic effect on the environment such as reduction in fresh water supply, erosion, and subsequent habitat and biodiversity loss [5,6]. The irrigation of feed crops for cattle alone accounts for nearly 8% of global human water use [2].

Until 2020 meat demand is expected to increase highly in developing countries and slightly in developed countries [7,8]. To meet these increased meat demands of modern society, animals are kept intensively, and production is optimized disregarding the well-being of the animals. Much of the growing demand for animal products worldwide is being met by concentrated animal feeding operations, or factory farms [2,9]. Factory farms account for 67% of poultry meat production, 50% of egg production, and 42% of pork production [10]. Globally some 56 billion animals and birds are raised and slaughtered for food each year [11]. Herding of animals in confined spaces in unfavorable conditions is practiced. Many of world's 17 billion hens and meat chickens each live in an area that is less than the size of a sheet of paper [12]. Cattle in feedlots often stand knee-high in manure and arrive at slaughterhouses covered in feces [13]. The adaptability of animals is not high enough to cope with these unnatural conditions and high stress levels are observed, resulting in disease, abnormal behavior, and death [14]. Furthermore, such operations are increasingly located in or near cities in the developing world, making urban areas the center of industrial meat production in some countries. This extraordinary proximate concentration of people and livestock poses probably one of the most serious environmental and public health challenges for the coming decades [15]. The World Organization for Animal Health (OIE) estimates that not less than 60% of human pathogens and 75% of recent emerging diseases are zoonotic [2]. Another problem is that of animal

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disease epidemics and more serious threat is posed by the chicken flu, as this can lead to possible new influenza epidemics or even pandemics, which can kill millions of people [16]. Foodborne illnesses have become increasingly problematic with a sixfold increase in gastroenteritis and food poisoning in industrialized countries in the last 20 years [17] and the most common causes of foodborne diseases in EU, the United States, and Canada are contaminated meats and animal products [18–21]. Nutrition-related diseases, such as cardiovascular disease and diabetes, associated with overconsumption of animal fats are now responsible for a third of global mortality [22]. In addition, there is a problem of antibiotics being used as growth promoters for animals kept in intensive farming. In the United States, livestock consumes 70% of all antimicrobial drugs [23]. This use probably contributes to the emergence of multidrug-resistant strains of pathogenic bacteria [24].

Furthermore, livestock competes with humans for crops and grains as they detract more from food supply than they provide. Livestock consume 77 million tons of protein contained in feedstuff that could potentially be used for human nutrition, whereas only 58 million tons of protein are contained in food stuffs that livestock produce and in terms of energy the relative loss is much higher [2]. Thus current meat production methods have several health, environmental, and ethical issues associated with them [5,6]. Continuation of meat production as usual will lead to further environmental degradation and destruction of habitats. However, solutions are within reach, many of which are from the scientific sector, and will need investment in form of time and money, and possibly changes in consumer's habits. Scientific innovations can and should come from all sectors involved, and a significant contribution can be made through generation of meat alternatives using improvements of already existing concepts and products. An example of such a concept is biofabrication, that is, production of complex living and nonliving biological products. A new approach to produce meat is probably feasible with existing tissue engineering techniques and has been proposed as a humane, safe, and environmentally beneficial alternative to slaughtered animal flesh. The industrial potential of biofabrication technology is far beyond the traditional medically oriented tissue engineering and organ printing, and, in the long term, it can contribute to the development of novel biotechnologies that can dramatically transform traditional animal-based agriculture by inventing "animal-free" food, leather, and fur products. The techniques required to produce cultured meat are not beyond imagination, and the basic methodology involves culturing muscle tissue in a liquid medium on a large scale; however, the production of highly structured unprocessed meat faces considerably greater technical challenges, and a great deal of research is still needed to establish a sustainable in vitro meat culturing system on an industrial scale. In the long term, cultured meat is an inescapable future of the humanity. In the short term, extremely high prohibitive cost of meat biofabrication is the main potential obstacle, although large-scale production and market penetration are usually associated with a dramatic price reduction. This chapter discusses various technological, regulatory, and ethical aspects associated with cultured meat production. A detailed attention has been paid to the challenges that need to be met for commercial feasibility and success of cultured meat production, which include finding an appropriate stem cell source, their growth in a three-dimensional environment inside a bioreactor, providing essential cues for proliferation and differentiation and evolving new processing technologies.

Need and advantages of cultured meat

1. This novel production system will significantly reduce the animal use as theoretically a single farm animal could be used to produce world's meat supply. Cultured meat has the potential to greatly reduce animal suffering and make eating animals unnecessary while satisfying all the nutritional and hedonic requirements of meat eaters [25]. In comparison with animals a product from a bioreactor could be attractive as it does not come with all the vicissitudes of animals. Cultured meat is potentially a much more reliable alternative and is not bound to soil or place, which opens possibilities for new places of production and for alternative land use. Considering cyanobacteria as a source of energy and nutrients, this production system has the potential to reduce 82%–96% of water usage, 78%–96% GHGs, and 99% land use associated with traditional meat production [26]. However, from a perspective of social acceptance, the technological character of cultured meat can have a negative value and associations with Frankenstein, cloning, transgenesis, and unknown risks are close at hand.
2. In theory, cells from captive rare or endangered animals (or even cells from samples of extinct animals) could be used to produce exotic meats in cultures as global trade of meats from rare and endangered animals has reduced wild populations of several species in many countries.
3. The chances of meat contamination and the incidence of foodborne diseases should be lower in an in vitro meat production system due to comprehensive monitoring systems and strict quality control rules, such as good manufacturing practice, that are impossible to introduce in modern animal farms, slaughterhouses, or meat packing plants. In addition, other risks such as exposure to pesticides,

arsenic, dioxins, and hormones associated with conventional meat should be significantly reduced.

4. With advent of functional and designer foods, consumers are more willing to try products that have been altered to have particular nutritional characteristics [27,28]. There are several possibilities to alter the functional value and nutritional profile of cultured meat to produce designer meat. The flavor and fatty acid composition of cultured meat could be influenced by manipulating the composition of the culture medium. Health aspects of the meat could be enhanced by adding factors such as certain types of vitamins to the culture medium, which might have an advantageous effect on health [29]. Coculturing with other cell types could enhance the meat quality. The ratio of saturated to polyunsaturated fatty acids could be better controlled, although the fat content could also be controlled by supplementation of fats after production.
5. Only muscle tissues will be developed in an in vitro meat production system and no other biological structures such as bones, respiratory system, digestive system, nervous system, and the skin will be involved, which should significantly reduce the amount of nutrients and energy needed for growth and maintenance of muscle tissue.
6. It takes several weeks (for chickens) or years (for pigs and cows) before the meat can be harvested in the current meat production systems. It will take significantly lower amount of time to grow the meat in vitro. Since the amount of the time needed to maintain the tissue is much lower in an in vitro meat production system, the amount of nutrients and labor required per kg of cultured meat should be much lower.
7. Since bioreactors for cultured meat production do not need extra space and could be stacked up in a fabric hall, the nutritional costs for cultured meat should be significantly lower than conventional meat. The financial advantages are not clear yet, and it might very well be that the decrease in costs of resources, labor, and land will be compensated by extra costs of a stricter hygiene regime, stricter control, computer management, etc.

   Although cultured meat is believed to significantly reduce the water and carbon foot print associated with meat production, the energy use associated with this novel production system is not clear yet and is expected to be more than poultry but lower than beef and possibly pork [26,30]. While comparing the environmental impact of different meat alternatives in a life cycle assessment, Smetana et al. [31] reported that cultured meat had higher impact on most categories when compared to other alternatives (plant-based, mycoprotein-based, and dairy-based) and chicken due to its higher level of energy requirements. However, the energy consumption associated with cultured meat production could be reduced by employing several innovative options in future.
8. There are several situations when it is costly to resupply people with food and it is more economical to produce food in situ. These include scientific stations in polar regions, troop encampments in isolated theaters of war and bunkers designed for long-term survival of personnel following a nuclear or biological attack. The long-term space missions such as a settlement on the Moon or a flight to Mars will likely involve some food production in situ within a settlement or spacecraft to reduce liftoff weight and its associated costs.
9. Need for other protein sources to meet the protein requirements of increasing population also demands the production of cultured meat. A definite market is available for meat substitutes, examples are legume-based and mycoprotein-based meat substitutes. Cultured meat may be a preferred alternative because it is, unlike other products, animal-derived and with respect to composition most like meat. A small market comprising the vegetarians that do not eat meat for ethical reasons may also be available.

   Demand for meat is increasing both in developing as well as developed countries, and it will not be possible to produce all the meat in an environmental- and animal-friendly way. Thus there is rather a conventional meat market for in vitro meat.
10. Cultured meat is expected to liberate itself of religious associations, such as *Kosher* and *Halal*, making it a universal product equally acceptable to whole spectrum of consumers. This could be an advantage for its marketing and should likely affect its consumer base. The concept of cultured meat has intrigued all religious bodies and institutions and several debates and discussion were held recently about the acceptability of this novel source of meat with favorable outcomes. Several Muslim scholars have suggested *Halal* nature of cultured meat provided the cells to begin with were harvested from *Halal* animals (permissible by the Islamic law) and all culture media components were *Halal* [32]. Although there is no consensus due to disparate nature of certifying bodies, most rabbis agree that cultured meat will be Kosher (permissible by Jewish laws) if cells were harvested from a kosher animal slaughtered according to Jewish law [33–35].
11. Other factors such as possible impact on reducing cardiovascular diseases and GHG emissions, liberation of land for nature (including wild animals), prevention of animal suffering, and prevention of food scarcity expected with an increasing world population will push the production of cultured meat in future.

Cultured meat

The idea of cultured meat for human consumption is not new but was predicted long back by Winston Churchill in the 1920s. In essay "Fifty Years Hence" later published in "Thoughts and adventures" in 1932, he declared, "Fifty years hence, we shall escape the absurdity of growing a whole chicken in order to eat the breast or wing by growing these parts separately under a suitable medium." In 1912 Alexis Carrel managed to keep a piece of chick heart muscle alive and beating in a Petri dish. This experiment demonstrated that it was possible to keep muscle tissue alive outside the body, provided it was nourished with suitable nutrients. It was much later in the early 1950s when Willem van Eelen of The Netherlands independently had the idea of using tissue culture for the generation of meat products. Since at that time the concept of stem cells and the in vitro culture of cells still had to emerge, it took until 1999 before van Eelen's theoretical idea was patented. Some efforts have already been put into culturing artificial meat. SymbioticA harvested muscle biopsies from frogs and kept the tissues alive and growing in culture dishes [36]. In 2002 a study involving the use of muscle tissue from common goldfish (*Carassius auratus*) cultured in Petri dishes was published in which the possibilities of culturing animal muscle protein for long-term space flights or habituation of space stations were explored. In this study, muscle tissue cultured with crude cell extracts showed a limited increase in cell mass and the cultured muscle explants so obtained were washed, dipped in olive oil with spices, covered in breadcrumbs, and fried. A test panel judged the processed explants and agreed that the product was acceptable as food [37]. Thus some efforts have already been put into culturing artificial meat; but obviously, small biopsies will not be practical for large-scale meat production. Therefore it was proposed to use tissue engineering to produce meat in vitro. Tissue engineering is a powerful technique used to mimic neo-organogenesis ex vivo and is mainly designated for regenerative medicine in a wide variety of tissues and organs for the treatment of various diseases and surgical reconstruction [38,39]. Tissue engineering of skeletal muscle has several applications, ranging from in vitro model systems for drug screening [40], pressure sores [41], and physiology to in vivo transplantation for the treatment of muscular dystrophy and muscular defects [42]. Obviously, tissue engineering could also be employed for in vitro production of skeletal muscle tissue from farm animals for consumption purposes [43].

Given the benefits of an in vitro meat production system, it is not surprising that several parties have proposed and patented the methodology for actualizing this idea [29,43,44]. However, none of these processes, though detailed, have been tested, this chapter introduces the techniques so far proposed. This is partially because farm-animal cell lines have not been well-established in vitro [45] and because growing muscle cells in vitro on a large scale is certainly a vast and unexplored undertaking. The technical demands of large-scale production are unseen in the world of medical research where most efforts in growing tissue ex vivo have been directed. The nutritional composition of ex vivo engineered muscle tissue has not yet been paid much attention. As a result, establishment of an in vitro meat production system is faced with many unique challenges so far unexplored in the field of tissue engineering.

The only aim to develop an in vitro meat production system is the proliferation of animal muscle tissue. Meat is already cultured on small and early scales using a variety of basic procedures, including techniques that use scaffolds and those that rely on self-organization [43]. There are different design approaches for an in vitro meat production system, all of which are designed to overcome the diffusion barrier, ranging from those currently in use to other speculative possibilities.

Scaffolding techniques

In scaffold-based techniques, embryonic myoblasts or adult skeletal muscle satellite cells are proliferated, attached to a scaffold or carrier such as a collagen meshwork or microcarrier beads, and then perfused with a culture medium in a stationary or rotating bioreactor. By introducing a variety of environmental cues, these cells fuse into myotubes, which can then differentiate into myofibers [46]. The resulting myofibers may then be harvested, cooked, and consumed as meat (Figs. 73.1 and 73.2).

Currently, there are two detailed proposals based on emerging field of tissue engineering [47,48] for using cell culture for producing in vitro meat. Both these proposals are similar in nature. One of the two proposals to create an in vitro meat production system has been written by Vladimir Mironov for the NASA [49] while the other proposal has been written by Willem van Eelen who also holds a worldwide patent for this system [29]. However, Catts and Zurr [36] appear to have been the first to have produced meat by this method. Both systems work by growing myoblasts in suspension in a culture medium. Mironov proposal uses a bioreactor in which cells are grown together with collagen spheres to provide a substrate onto which the myoblasts can attach and differentiate, whereas van Eelen's proposal uses a collagen meshwork and the culture medium is refreshed from time to time or percolated through the meshwork. Once differentiated into myofibers, the mixture of collagen and muscle cells can be harvested and used as meat. Other forms

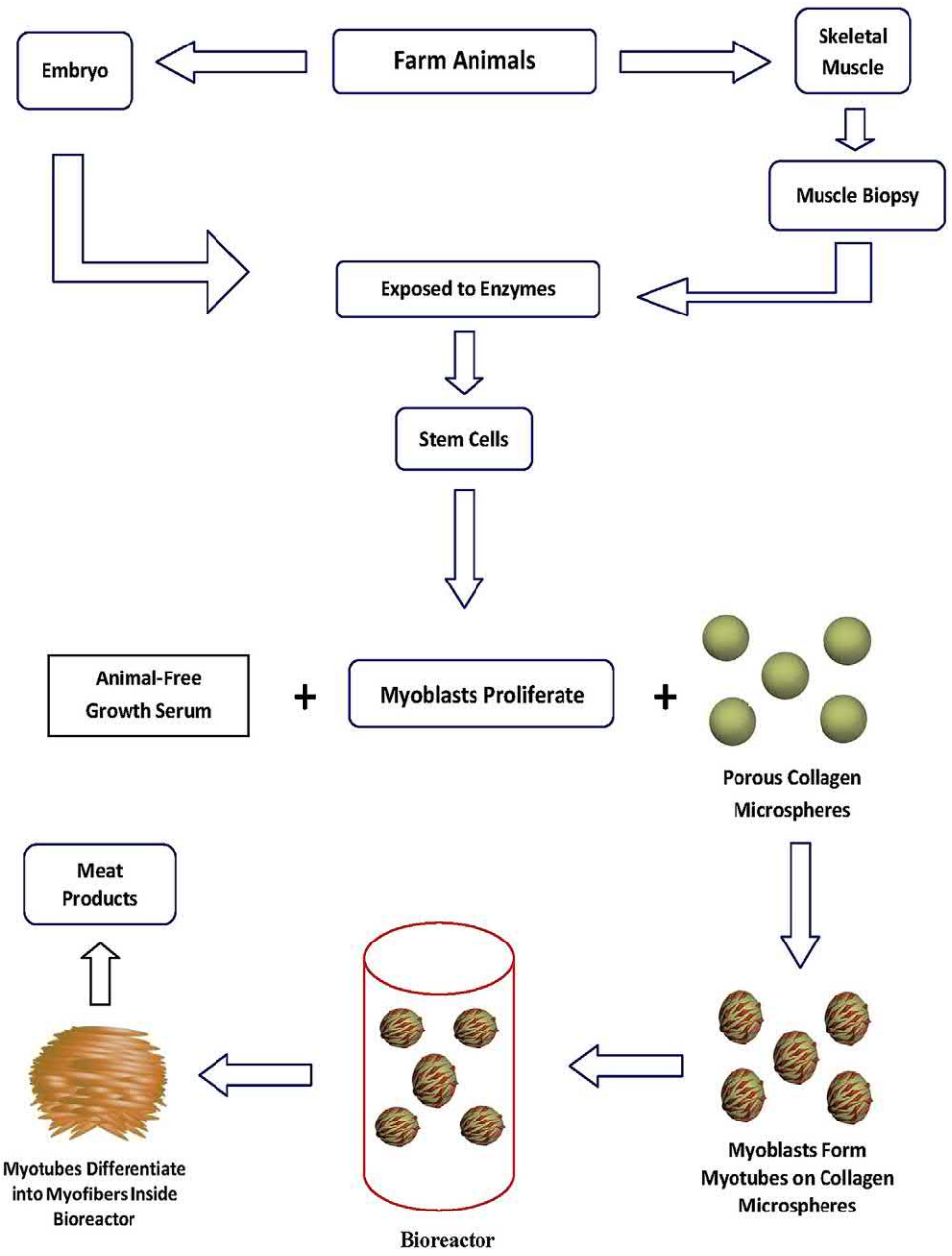

FIGURE 73.1 Scaffold-based cultured meat.

of scaffolding could also be used, for example, growing muscle tissue on large sheets of edible or easily separable material. The muscle tissue could be processed after being rolled up to suitable thicknesses [43]. While these kinds of techniques work for producing ground processed (boneless) meat with soft consistency, they do not lend themselves to highly structured meats such as steaks. However, cells can also be grown in substrates that allow for the development of "self-organizing constructs" that produce more rigid structures.

Self-organizing tissue culture

To produce highly structured meats, one would need a more ambitious approach, creating structured muscle tissue as self-organizing constructs [50] or proliferating existing muscle tissue in vitro, such as Benjaminson et al. [37] who cultured goldfish (*C. auratus*) muscle explants. They took slices of goldfish tissue, minced and centrifuged them to form pellets, placed them in Petri dishes in a nutrient medium and grew them for 7 days. The explanted tissue grew nearly 14% when using fetal bovine

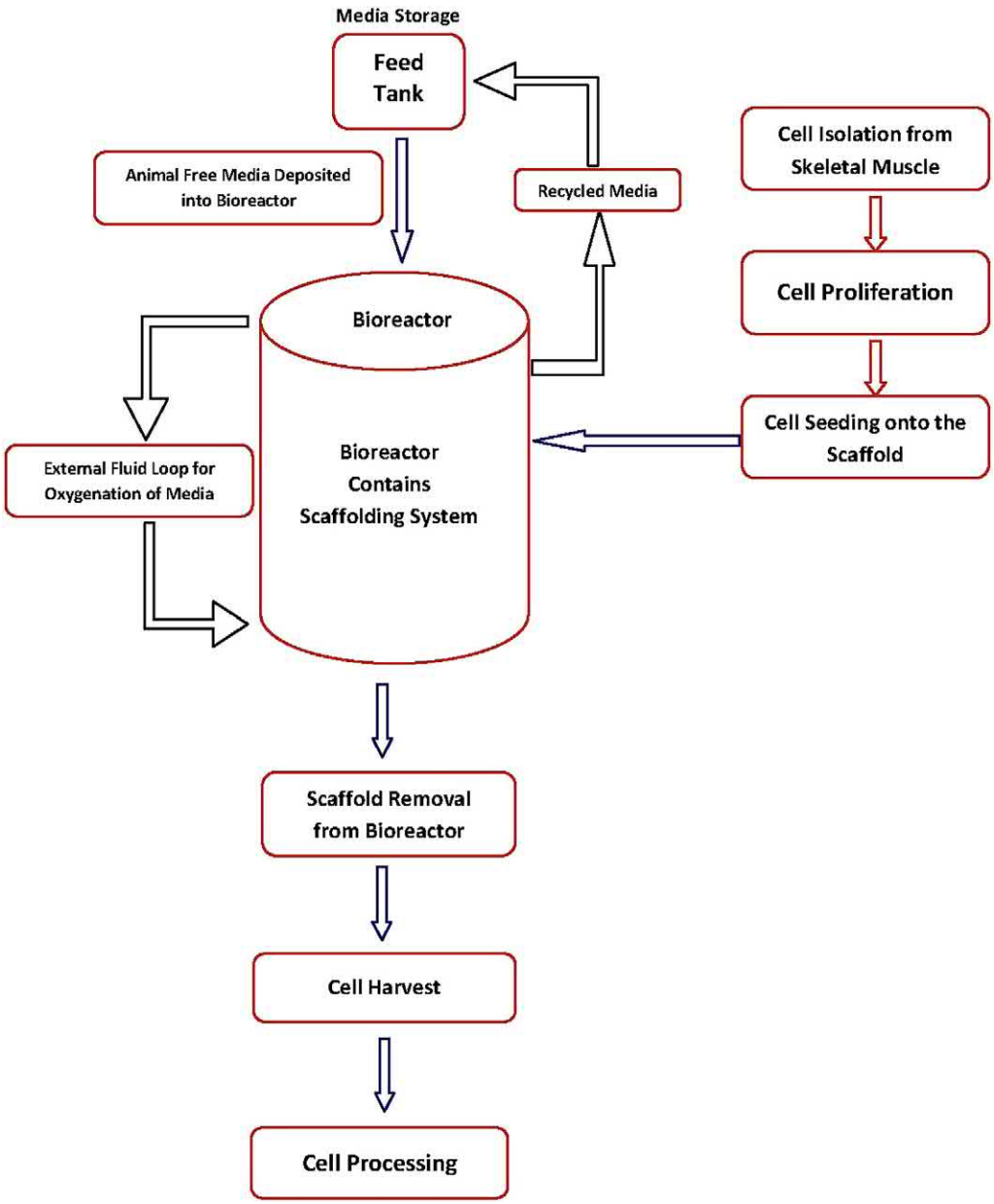

FIGURE 73.2 Possible in vitro meat production scheme.

serum as the nutrient medium and over 13% when using maitake mushroom extract. When the explants were placed in a culture containing dissociated *Carassius* skeletal muscle cells, explant surface area grew a surprising 79% in a week's time. After a week the explants and their newly grown tissue, which looked like fresh fish filets, were cooked (marinated in olive oil and garlic and deep-fried) and presented to a panel for observation. The panel reported that the fish looked and smelled good enough to eat [37,51–53]. Benjaminson et al. in another experiment kept chicken muscles alive in a Petri dish for at most 2 months before it got necrosed [49].

Tissue culture techniques have the advantages that explants contain all the tissues that make up meat in the right proportions and closely mimic in vivo situation. However, lack of blood circulation in these explants makes substantial growth impossible, as cells become necrotic if separated for long periods by more than 0.5 mm from a nutrient supply [50]. According to Vladimir Mironov, entirely artificial muscle can be created with tissue engineering techniques by a branching network of edible porous polymer through which nutrients can perfuse and myoblasts and other cell types can attach [49]. Such a design using the artificial capillaries for the

purpose of tissue engineering has been proposed [48]. Small muscle–like organs have been demonstrated to grow from cocultures of myoblasts and fibroblasts. These organs, termed myooids, can contract both spontaneously and by electrical stimulation, albeit with only a fraction of the force observed in control muscles probably because of lack of innervation [50,54,55]. The diameter of myooids is limited at most to 1 mm [55] due to the lack of perfusion, which is probably the biggest problem to overcome in designing an in vitro meat production system. Like the myooids, it is possible to coculture the myoblasts with other cell types to create a more realistic muscle structure that can be organized in much the same way as real muscles [50,54,55].

Organ printing

The problems associated with current tissue engineering techniques are that they cannot provide consistency, vascularization, fat marbling, or other elements of workable and suitably tasting meat that are not simply versions of ground soft meat. A potential solution to such problems comes from research on producing organs for transplantation procedures known as organ printing. Organ printing uses the principles of ordinary printing technology, the technology used by inkjet printers to produce documents. Researchers use solutions containing single cells or balls of cells and spray these cell mixtures onto gels that act as printing paper. The "paper" can be removed through a simple heating technique or could potentially be automatically degradable. What happens is essentially that live cells are sprayed in layers to create any shape or structure desired. After spraying these three-dimensional structures, the cells fuse into larger structures, such as rings, tubes, or sheets. The organs would not only have the basic cellular structure of the organ but also include appropriate vascularization providing a blood supply to the entire product. Essentially, sheets and tubes of appropriate cellular components could create any sort of organ or tissue for transplantation or for consumption [25,56,57]. For applications focused on producing meat, fat marbling could be added as well, providing taste and structure.

Biophotonics

Biophotonics is a new field that relies on the effects of lasers to move particles of matter into certain organizational structures, such as three-dimensional chessboard or hexagonal arrays. In general, biophotonics refers to the process of using light to bind together particles of matter, and the mechanisms of this field are still poorly understood. A surprising property of interacting light is that this phenomenon produces so-called optical matter in which the crystalline form of materials (such as polystyrene beads) can be held together by nets of infrared light that will fall apart when the light is removed. This is a phenomenon a step-up from "optical tweezers" that have been used for years to rotate or otherwise move tiny particles in laboratories. This has a binding effect among a group of particles that can lead them not only to be moved one by one to specific locations but also that can coax them to form structures. Although primarily there is sparking interest in medical technologies such as separating cells or delivering medicine or other microencapsulized substances to individual cells, there is an intriguing possibility that such a technology could be used to produce tissues, including meat [25]. Arrays of red blood cells and hamster ovaries have already been created using this technology [58]. Given the success of creating two-dimensional arrays, there is a possibility of producing tissue formations that use only light to hold the cells together, thus eliminating the need for scaffoldings [25].

Nanotechnology

The ability of optical tweezers to rotate or move tiny particles has intrigued nanotechnologists, who have inventive plans for what to do with the molecular scale–sized robots they would like to create (but so far, having few tools with which to make them). Nanotechnology (the production and alteration of materials at the level of the atom and molecule) holds out enormous possibilities and the holy grail of nanotechnology is some version of an "assembler," a robot the size of a molecule that would allow moving matter at the atomic and molecular level. The obvious power of such a technology, given that everything is made of the same basic atoms but simply arranged in different ways, is that we would be able to construct virtually any substance we wanted from scratch by putting together exactly the molecules we wanted. Interestingly, one of the first examples given of the speculative technology of nanotechnology was that of synthesized meat. Thus technologies ranging from the actual to the speculative promise a variety of ways to create real meat without killing animals. Though commercially not feasible now or in some cases technologically infeasible for several years to come, the point here is not to be distracted by the fact that we cannot yet make use of these technologies but rather to decide whether we should support the development of these technologies [25].

Challenges and requirements for industrial production

There are two different ideas regarding the concept of cultured meat. Because people like meat and cultured meat is explicitly introduced as an alternative to the problems of normal meat, it should be as meat-like as possible in

order to be a real alternative for "traditional" meat from animals. It is therefore important that an alternative should have a similar taste and nutritional value. On the other hand, a new product needs a profile of its own otherwise it will not be able to compete. From this perspective, it is not essential for the product to resemble and should in fact be clearly distinctive from traditional meat.

Cultured meat technology is still in its infancy, and the most important challenge is insufficient knowledge on the biology of the stem cells and its differentiation into muscle cells. Tissue engineering on a very large scale is the second requirement along with the maintenance of constant conditions around all individual cells in a large-scale reactor with sophisticated instrumentation for measuring and controlling conditions. Need of cell growth and differentiation and subsequent release from support without damage upon harvesting is third requirement along with the need for on-site cleaning and sterilization systems in the large-scale reactors. Studies are required to determine the consumer preferences and marketing strategies. When meat from animals is available why would a consumer prefer cultured meat and if it is all about sustainability or animal welfare issues, eating more plant proteins and less animal protein is a good alternative. The following challenges have to be met before cultured meat can be produced on a commercial scale.

Generation of suitable stem cell lines from farm-animal species

In vitro meat can be produced by culturing the cells from farm-animal species in large quantities starting from a relatively small number. Culturing embryonic stem (ES) cells would be ideal for this purpose since these cells have an almost infinite self-renewal capacity and theoretically it is being said that one such cell line would be sufficient to feed the world. In theory, after the ES cell line is established, its unlimited regenerative potential eliminates the need to harvest more cells from embryos; however, the slow accumulation of genetic mutations over time may determine a maximum proliferation period for a useful long-term ES culture [59]. While ES cells are an attractive option for their unlimited proliferative capacity, these cells must be specifically stimulated to differentiate into myoblasts and may inaccurately recapitulate myogenesis [39]. Although ES cells have been cultured for several generations, so far it has not been possible to culture cell lines with unlimited self-renewal potential from preimplantation embryos of farm-animal species. Until now, true ES cell lines have only been generated from mouse, rhesus monkey, human, and rat embryos, [45] but the social resistance to cultured meat obtained from mouse, rat, or rhesus monkey will be considerable and will not result in a marketable product. The culture conditions required to keep mouse and human embryonic cells undifferentiated are different from the conditions that will be required for embryonic cells of farm-animal species and fundamental research on early development of embryos of these species can provide clues.

Different efforts invested into establishing ungulate stem cell lines over the past two decades have been generally unsuccessful with difficulties arising in the recognition, isolation, and differentiation of these cells [60]. According to Bach et al. [39], myosatellite cells are the preferred source of primary myoblast; however, they have the disadvantage of being a rare muscle tissue cell type with limited regenerative potential because they recapitulate myogenesis more closely than immortal myogenic cell lines. Myosatellite cells isolated from different animal species have different benefits and limitations as a cell source and those isolated from different muscles have different capabilities to proliferate, differentiate or being regulated by growth modifiers [61]. Myosatellite cells have been isolated and characterized from the skeletal muscle tissue of cattle [62], chicken [63], fish [64], lambs [65], pigs [66], and turkeys [67]. Porcine muscle progenitor cells have the potential for multilineage differentiation into adipogenic, osteogenic, and chondrogenic lineages, which can play a role in the development of cocultures [66]. Advanced technology in tissue engineering and cell biology offers some alternate cell options having practical applications and multilineage potential allowing for coculture development with suitability for large-scale operations.

Alternatively, we can use adult stem cells from farm-animal species, and myosatellite cells are one example of an adult stem cell type with multilineage potential [68]. Adult stem cells have been isolated from different adult tissues [69]; however, their proliferation capacity is limited in vitro and could proliferate for several months at most. These cells have the capacity to differentiate into skeletal muscle cells although not very efficiently but for now these are the most promising cell type for use in the production of cultured meat. A rare population of multipotent cells found in adipose tissue known as adipose tissue−derived adult stem cells (ADSCs) is another relevant cell type for cultured meat production [70], which can be obtained from subcutaneous fat and subsequently transdifferentiated to myogenic, osteogenic, chondrogenic, or adipogenic cell lineages [71]. However, adult stem cells are prone to malignant transformation in long-term culture [72] that is the greatest matter of debate. It has been observed that ADSCs immortalize at high frequency and undergo spontaneous transformation in long-term (4−5 months) culturing [73], while evidence of adult stem cells remaining untransformed has also been reported [74]. To minimize the risk of spontaneous transformation,

reharvesting of adult stem cells may be necessary in an in vitro meat production system and as such obtaining ADSCs from subcutaneous fat is far less invasive than collection of myosatellite cells from muscle tissue.

Matsumoto et al. [75] reported that mature adipocytes can be dedifferentiated in vitro into a multipotent preadipocyte cell line known as dedifferentiated fat (DFAT) cells, reversion of a terminally differentiated cell into a multipotent cell type. These DFAT cells are capable of being transdifferentiated into skeletal myocytes [76] and appear to be an attractive alternative to the use of stem cells. This process known as "ceiling culture method" certainly seems achievable on an industrial scale but Rizzino [77] has put forth the argument that many of the claims of transdifferentiation, dedifferentiation, and multipotency of once terminally differentiated cells may be due to abnormal processes resulting in cellular look-alikes.

Safe media for culturing of stem cells

Cultured meat would need an affordable medium system to enjoy its potential advantages over conventional meat production and that medium must contain the necessary nutritional components available in free form to myoblasts and accompanying cells. Myoblast culturing usually takes place in animal serum, a costly medium that does not lend itself well to consumer acceptance or large-scale use. Animal sera are from adult, newborn or fetal source, with fetal bovine serum being the standard supplement for cell culture media [78]. Because of its in vivo source, it can potentially introduce pathogenic agents and have a large number of constituents in highly variable composition [79]. The harvest of fetal bovine serum also raises ethical concern; and for generation of an animal-free protein product, the addition of fetal calf serum to the cells would not be an option and it is therefore essential to develop a serum-free culture medium. Commercially available serum replacements and serum-free culture media offer some more realistic options for culturing mammalian cells in vitro. Serum-free media reduce operating costs and process variability while lessening the potential source of infectious agents [80]. Improvements in the composition of commercially available cell culture media have enhanced our ability to successfully culture many types of animal cells and serum-free media have been developed to support in vitro myosatellite cell cultures from the turkey [81], sheep [82], and pig [83]. Variations among different serum-free media outline the fact that satellite cells from different species have different requirements and respond differentially to certain additives [84]. Ultroser G is an example of a commercially available serum substitute containing growth factors, binding proteins, adhesin factors, vitamins, hormones, and mineral trace elements and has been designed specially to replace fetal bovine serum for growth of anchorage-dependent cells in vitro [85]. Benjaminson et al. [37] succeeded in using a serum-free medium made from maitake mushroom extract that achieved higher rates of growth than fetal bovine serum, and recently it has been shown that lipids such as sphingosine-1-phosphate can replace serum in supporting the growth and differentiation of embryonic tissue explants. In most cases, serum-free media are supplemented with purified proteins of animal origin [86].

Indeed, such media have already been generated and are available from various companies for biomedical purposes; however, their price is incompatible with the generation of an affordable edible product. Therefore a cell culture medium must be developed that does not contain products of animal origin and enables culturing of cells at an affordable price.

Safe differentiation media to produce muscle cells

For culturing stem cells, it is important that the cells remain undifferentiated and maintain their capacity to proliferate, whereas for production of cultured meat, a specific and efficient differentiation process initiated with specific growth factors is needed. These growth factors are synthesized and released by muscle cells themselves and are also provided by other cell types locally (paracrine effects) and nonlocally (endocrine effects) in vivo. An appropriate array of growth factors is required for growth of muscle cells in culture in addition to proper nutrition. The myosatellite cells of different species respond differentially to the same regulatory factors [61] and as such extrinsic regulatory factors must be specific to the chosen cell type and species. Furthermore, formulation may be required to change over the course of the culturing process from proliferation period to the differentiation and maturation period, requiring different set of factors. A multitude of regulatory factors have been identified as being capable of inducing myosatellite cell proliferation [87]. Regulation of meat animal−derived myosatellite cells by hormones, polypeptide growth factors, and extracellular matrix proteins has been investigated [83,84]. Purified growth factors or hormones may be supplemented into the media from an external source such as transgenic bacterial, plant, or animal species, which produce recombinant proteins [88]. Alternatively, a sort of synthetic paracrine signaling system can be arranged so that cocultured cell types can secrete growth factors that can promote proliferation and cell growth in neighboring cells. Appropriate coculture systems, such as hepatocytes, may be developed to provide necessary growth factors, such as insulin-like growth factors, which

will stimulate myoblast proliferation and differentiation [89] as well as myosatellite cell proliferation in several meat-animal species in vitro [84]. Typically, investigators initiate differentiation and fusion of myoblasts by lowering the levels of mitogenic growth factors, and the proliferating cells then commence synthesis of insulin-like growth factor-II, which leads to differentiation and formation of multinucleated myotubes [90] and stimulate myocyte maturation [91]. Thus a successful system must be capable of changing the growth factor composition of the media. Currently, the most efficient method to let stem cells (mouse) differentiate into skeletal muscle cells is to culture them in a medium that contains 2% horse serum instead of 10% or 20% fetal calf serum. However, for the generation of cultured meat, it is essential that the cells are cultured and differentiated without animal products, so a chemically defined culture medium must be developed that enables the differentiation of stem cells to skeletal muscle cells.

Tissue engineering of muscle fibers

The possibility to form a three-dimensional structure of cells is restricted in the absence of blood flow that provides oxygen and nutrients to the cells and removes metabolic end products. Because of the limitations in nutrient diffusion, in vitro culturing of cells is limited to only a few layers of cells. A solution to this problem may be provided by culture of cells on edible or biodegradable synthetic or biological scaffolds, which would provide shape and structure to the engineered tissue. Another solution could be the processing of these thin layers of cells into a meat-based product. Alternatively, deformable microcarrier beads of edible (nonanimal) material may be developed that would enable production of secondary myotubes in suspension, which may be used as an animal protein ingredient for a wide variety of products. Products with a meat-like appearance and texture could be developed by addition of fibroblasts (for firmness) and fat cells (for taste) to the myotubes.

Scaffolds

As myoblasts are anchorage-dependent cells, a substratum or scaffold must be provided for proliferation and differentiation to occur [92]. Scaffolding mechanisms differ in shape, composition, and characteristics to optimize muscle cell and tissue morphology. An ideal scaffold must have a large surface area for growth and attachment, be flexible to allow for contraction as myoblasts are capable of spontaneous contraction, maximize medium diffusion, and be easily dissociated from the meat culture. A best scaffold is one that mimics the in vivo situation as myotubes differentiate optimally on scaffold with a tissue-like stiffness [93] and its by-products must be edible and natural and may be derived from nonanimal sources, though inedible scaffold materials cannot be disregarded. New biomaterials may be developed that offer additional characteristics, such as fulfilling the requirement of contraction for proliferation and differentiation [94]. Thus challenge is to develop a scaffold that can mechanically stretch attached cells to stimulate differentiation and a flexible substratum to prevent detachment of developing myotubes that will normally undergo spontaneous contraction.

Edelman et al. [43] proposed porous beads made of edible collagen as a substrate while as van Eelen et al. [29] proposed a collagen meshwork described as a "collagen sponge" of bovine origin. The tribeculate structure of the sponge allows for increased surface area and diffusion but may impede harvesting of the tissue culture. Other possible scaffold forms include large elastic sheets or an array of long, thin filaments. Cytodex-3 microcarrier beads have been used as scaffolds in rotary bioreactors, but these beads have no stretching potential. One elegant approach to mechanically stretch myoblasts would be to use edible, stimuli-sensitive porous microspheres made from cellulose, alginate, chitosan, or collagen [43], which undergo, at least, a 10% change in surface area following small changes in temperature or pH. Once myoblasts attach to the spheres, they could be stretched periodically provided such variation in the pH or temperature would not negatively affect cell proliferation, adhesion, and growth. Jun et al. [95] observed that growing myoblasts on electrically conductive fibers induces their differentiation, forming more myotubes of greater length without the addition of electrical stimulation, but use of such inedible scaffolding systems necessitates simple and nondestructive techniques for removal of the culture from the scaffold.

Furthermore, there are greater technical challenges in developing a scaffold for large and highly structured meats due to the absence of vascular system. There is a need to build a branching network from an edible, elastic, and porous material, through which nutrients can be perfused and myoblasts and other cell types can then attach to this network. Edelman et al. [43] acknowledged that a cast of an existing vascularization network, such as that in native muscle tissue, can be used to create a collagen network mimicking native vessel architecture. Taking this a step further, Borenstein et al. [96] have proposed an approach to create such a network by creating a cast onto which a collagen solution or a biocompatible polymer is spread and after solidification seeding the network with endothelial cells. Following dissolution of the polymer mold, successful proliferation could theoretically leave behind a network of endothelial tissue, a branched network of micro-channels, which can be stacked onto each

other to form a three-dimensional network onto which one could grow myocytes. A synthetic vascular system would then require a circulation pumping system and a soluble oxygen carrier in the medium to be fully functional. However, currently creation of these artificial vascular networks does not translate well into mass production due to the requirement of microfabrication processes. Alternatively, Benjaminson et al. [37] proposed an attempt to create a highly structured meat without a scaffold by solving the vascularization problem through controlled angiogenesis of explants.

Another important factor is the texture and microstructure of scaffolds as texturized surfaces can attend to specific requirements of muscle cells, one of which is myofiber alignment. This myofiber organization is an important determinant for the functional characteristics of muscle and the textural characteristics of meat. Lam et al. [97] cultured myoblasts on a substrate with a wavy micropatterned surface to mimic native muscle architecture and found that the wave pattern aligned differentiated muscle cells while promoting myoblast fusion to produce aligned myotubes. While using scaffold-based techniques for meat culturing, micropatterned surfaces could allow muscle tissue to assume a two-dimensional structure more similar to that of conventional meat. Riboldi et al. [98] utilized electrospinning, a process that uses electrical charge to extract very fine fibers from liquids, by using electrospun microfibrous meshwork membranes as a scaffold for skeletal myocytes. These membranes offer high surface area−to-volume ratio in addition to some elastic properties. Electrospinning creates very smooth fibers, which may not translate well into a good adhesive surface; however, coating electrospun polymer fibers with extracellular matrix proteins, such as collagen or fibronectin, promotes attachment by ligand−receptor binding interactions [98]. Electrospinning shows promise for scaffold formation because the process is simple, controllable, reproducible, and capable of producing polymers with special properties by cospinning [98].

Production of meat by scaffold-based techniques also faces a technical challenge of removal of the scaffolding system. Confluent cultured cell sheets are conventionally removed enzymatically or mechanically, but these two methods damage the cells and the extracellular matrix they may be producing [99]. However, thermoresponsive coatings that change from hydrophobic to hydrophilic at lowered temperatures can release cultured cells and extracellular matrix as an intact sheet upon cooling [100]. This method known as “thermal liftoff” results in undamaged sheets that maintain the ability to adhere if transferred onto another substrate [100] and opens the possibility of stacking sheets to create a three-dimensional product. Lam et al. [101] have presented a method for detaching culture as a confluent sheet from a nonadhesive micropatterned surface using the biodegradation of selective attachment protein laminin. However, culturing on a scaffold may not result in a two-dimensional confluent “sheet” of culture. The contractile forces exerted after scaffold removal by the cytoskeleton of the myocyte are no longer balanced by adhesion to a surface that causes the cell lawn to contract and aggregate, forming a detached multicellular spheroid [100]. To remove the culture as a sheet, a hydrophilic membrane or gel placed on the apical surface of the culture before detachment can provide physical support and use of a fibrin hydrogel is ideal for skeletal muscle tissue because cells can migrate, proliferate, and produce their own extracellular matrix within it while degrading excess fibrin [101]. These two-dimensional sheets could be stacked to create a three-dimensional product as suggested by van Eelen et al. [29].

Industrial bioreactors

Production of cultured meat for commercial purposes will require large-scale culturing in large bioreactors for the generation of sufficient number of muscle cells. Cultured meat production is likely to require the development of new bioreactors, which would maintain low shear and uniform perfusion at large volumes. The bioreactor designing is intended to promote the growth of tissue cultures that accurately resemble native tissue architecture and provide an environment, which allows for increased culture volumes. A laminar flow of the medium is created in rotating wall vessel bioreactors by rotating the cylindrical wall at a speed that balances centrifugal force, drag force, and gravitational force, leaving the three-dimensional culture submerged in the medium in a perpetual free-fall state [102] that improves diffusion with high mass transfer rates at minimal levels of shear stress, producing three-dimensional tissues with structures very similar to those in vivo [103]. Direct perfusion bioreactors appear more appropriate for scaffold-based myocyte cultivation allowing flow of medium through a porous scaffold with gas exchange taking place in an external fluid loop [104]. Besides offering high mass transfer they also offer significant shear stress, so determining an appropriate flow rate is essential [103]. Direct perfusion bioreactors are also used for high-density, uniform myocyte cell seeding [105]. Another method of increasing medium perfusion is by vascularizing the tissue being grown. Levenberg et al. [106] induced endothelial vessel networks in skeletal muscle tissue constructs by using a coculture of myoblasts, embryonic fibroblasts, and endothelial cells coseeded onto a highly porous biodegradable scaffold. Research size bioreactors for mesenchymal stem cells have been scaled up to 5 L [107] and, theoretically,

scale-up to industrial sizes should not affect the physics of the system.

Adequate perfusion of the cultured tissue is required to produce large culture quantities, and it is necessary to have adequate oxygen perfusion during cell seeding and cultivation on the scaffold as cell viability and density positively correlate with the oxygen gradient in statically grown tissue cultures [105]. Adequate oxygen perfusion is mediated by bioreactors that increase mass transport between culture medium and cells and by the use of oxygen carriers to mimic hemoglobin provided oxygen supply to maintain high oxygen concentrations in solution similar to that of blood. Oxygen carriers are either modified versions of hemoglobin or artificially produced perfluorochemicals that are chemically inert [108]. Several chemically modified hemoglobins have been developed, but their bovine or human source makes them an unsuitable candidate for cultured meat production; however, hemoglobins have also been produced from genetically modified plants [109] and microorganisms [110].

Fields

Proliferation and differentiation of myoblasts have been found to be affected by the mechanical, electromagnetic, gravitational, and fluid flow fields [46,94]. Repetitive stretch and relaxation equal to 10% of length, six times per hour increase differentiation into myotubes [111]. Myoblasts seeded with magnetic microparticles induced differentiation by placing them in a magnetic field without adding special growth factors or any conditioned medium [112]. Electrical stimulation also contributes to differentiation, as well as sarcomere formation within established myotubes [46].

Atrophy and exercise

One of the potential problems associated with cultured meat is that of atrophy or muscle wasting due to the reduction of cell size [113] caused by lack of use, denervation, or one of a variety of diseases [114,115]. Regular contraction is a necessity for skeletal muscle and promotes differentiation and healthy myofiber morphology while preventing atrophy. Muscle in vivo is innervated, allowing for regular, controlled contraction, whereas in vitro system would necessarily culture denervated muscle tissue, so contraction must be stimulated by alternate means. It might be possible that mechanical or electrical stimulation can promote growth and structure of cultured meat as newly formed myotubes in culture start to contract spontaneously [116] or as a matter of fact, myooids also contract spontaneously at approximately 1 Hz once formed [50]. So, exercise by electrical stimulation might be a viable solution to overcome atrophy in an in vitro meat production system. Cha et al. [117] have found that administration of cyclic mechanical strain to a highly porous scaffold sheet promotes differentiation and alignment of smooth muscle cells. Edelman et al. [43] and van Eelen et al. [29] proposed mechanical stretching of scaffolds and expandable scaffold beads to fulfill the requirement of providing contraction. De Deyne [94] noted that external mechanical contraction is less effective than electrical stimulation in promoting muscle development. Electrical stimulation, feasible on a large scale, induces contraction internally as opposed to passively and aids in differentiation and sarcomere formation. Even growth on electrically conductive fibers without application of electrical stimulation sufficed in reaping the benefits of induced contraction [95].

Neuronal activity can be mimicked by applying appropriate electrical stimuli to in vitro cultures [118] and has proven to be pivotal in the development of mature muscle fibers [119]. It has been shown that induction of contractile activity promoted the differentiation of myotubes in culture by myosin heavy chain expression of different isoforms and sarcomere development [120,121]. Electrical stimulation can provide a noninvasive and accurate tool to assess the functionality of engineered muscle constructs [122]. Functional muscle constructs will exert a force due to active contractions of the muscle cells by generating a homogeneous electrical field inside the bioreactor; but so far, these forces generated by engineered muscle constructs only reach 2%−8% of those generated by skeletal muscles of adult rodents [54]. Thus functional properties of tissue-engineered muscle constructs are still unsatisfactory at this moment.

Mechanotransduction is the process through which cells react to mechanical stimuli and is a complex mechanism [123,124] that is another important biophysical stimulus in myogenesis [125]. It is mainly by means of the family of integrin receptors that cells attach to the insoluble meshwork of extracellular matrix proteins [126] transmitting the applied force to the cytoskeleton. The resulting series of events shows parallels to growth factor receptor signaling pathways, which ultimately lead to changes in cell behavior, such as proliferation and differentiation [123]. Muscle growth and maturation is affected by different mechanical stimulation regimes, and the application of static mechanical stretch to myoblasts in vitro results in a facilitated alignment and fusion of myotubes and also results in hypertrophy of the myotubes [125]. Furthermore, cyclic strain activates quiescent satellite cells [127] and increases proliferation of myoblasts [128]. Thus all these results indicate that mechanical stimulation protocols affect both proliferation and differentiation of muscle cells, and different parameters that presumably influence the outcome of the given stimulus are percentage of applied stretch, frequency of the stimulus, and timing in the differentiation process.

Senescence

Cultured meat production system based on satellite cells still imposes a challenge of senescence that can be tackled either by starting fresh cell culture whenever needed or by immortalizing cell culture or by using ES cell cultures. Fresh satellite cells can be extracted without harming the animal donors [29] from time to time to start new cell cultures, although animal slaughter is a more common practice [61]. Second approach involves modification of the cells in culture so that senescence can be overcome by involving the ectopic expression of the gene for the telomerase enzyme [129]. An additional expression of an oncogene may be required to overcome senescence [130–133], but this method falls within the domain of genetic modification, which might severely hinder consumer acceptance. Third approach involves ES cells that are pluripotent and apparently have an unlimited capability for division [134], and thus ES cell culture derived from a single donor can be theoretically propagated unlimited, but ES cells have to differentiate to muscle cells before they can be used.

Meat processing technology

Since three-dimensional fully structured meat is not possible with existing bioengineering technologies, the cultured meat is likely to be introduced in the form of comminuted meat products such as patties, nuggets, balls, and sausages. Cultured meat produced currently lacks in certain sensorial attributes, such as color, texture, and juiciness; and the processing phase will have to take care of these shortcomings and come up with nutritionally and sensorially acceptable meat products. New industrial-scale processing technologies are required to make cultured meat–based products more attractive and at least sensorially as acceptable as conventional meat products. Although an imaginary "The *in vitro* meat cookbook" was launched in 2014 that covered 45 different recipes [135], the research is totally lacking on product processing part and needs immediate scientific attention.

Associated dangers and risks

Since every new technology brings new risks and dangers with it, cultured meat may have a completely different risk profile than conventional meat. Much attention would require to be paid to the safety of added substrates and other compounds of the culture medium. Cultured meat may have some environmental advantages than conventional meat; however, production may be associated with some new risks of contamination. The meat will be cultured under sterile conditions within a closed bioreactor making sure that aseptic conditions are used during this phase of production. So, there are fewer risks with respect to microbial contamination but more risk of contamination of substrates. After the culturing phase of production, the cultured meat will be dealt with similar quality control and hygienic conditions as applicable to any regular standard meat settings. Thus during this phase, there will be similar risks and dangers as are expected in regular meat production and need to be dealt with accordingly.

Since more skilled personnel and perhaps robotics and automation would be required to manage this more sophisticated production system, it will certainly affect the job opportunities for less skilled persons associated with conventional meat industry. Although it is hard to evaluate the possible health hazards and risks associated with this production system beforehand, certain probable outcomes such as there will be lesser open lands in rural areas and more forests as fodder requirements will be significantly reduced. There will be for sure some favorable outcomes on animal welfare and environmental front. However, some of the areas such as genetic instability of multiple cell divisions [136] and the issues with media components [137] may require special attention. Future efforts in culturing meat will have to address the limitations of current techniques making cultured cells, scaffolds, culture media, and growth factors edible and affordable.

Regulatory issues

There are no regulatory guidelines issued for this new product because the production of cultured meat is currently limited to research level. Being essentially a consumable meat product, this new production system is likely to be overseen by the food safety authorities and is expected to vary from one country to the other and at least at par to regular standard meat production settings. Although there is also a possibility that the initial stages of the production involving the cell collection, maintaining the cell banks, and cell growth and differentiation, considered as a domain for medical expertise, could fall under the medical supervision. A comprehensive monitoring system needs to ensure that the process could be aborted in the case of deviations at the critical control points and start over the process a fresh. This production system may be more complicated than the conventional meat production; however, it inherits certain advantages in terms of meat safety for being grown under microbiologically and contamination-free environment.

Recently, a public meeting was held by the US Food and Drug Administration (FDA) and the US Department of Agriculture (USDA) to discuss the use of poultry and livestock cell lines to develop food products based on cell-cultured meat [138]. It was concluded and later announced that there will be a joint regulatory framework

and both the FDA and the USDA will jointly oversee the production of cell-cultured meat and meat products. The stages involved in the beginning of the production process such as cell collection, cell banks, and cell growth and differentiation will be monitored by the FDA, whereas the later stages involving the production and labeling of the cultured meat products will be overseen by the USDA, thus benefiting from the expertise of both the regulatory bodies, that is, the FDA's experience of handling living biosystems and cell-culture technology and the USDA's experience of regulating the meat production.

Consumer acceptance and perception

Several aspects of cultured meat have been studied by several researchers, including the acceptance and perception of consumers toward this novel product (Table 73.1). While Mattick and Allenby [139] analyzed the consequences of a potential shift from livestock agriculture to cultured meat, Hocquette et al. [140] studied the limitations associated with cultured meat such as economic, social, and technical constraints, also including the uncertain consumer acceptance. Post [141] identified public perception as the least studied aspect of cultured meat while highlighting the various technological challenges that lay ahead. While studying the consumers reaction and attitude formation to the cultured meat, Verbeke et al. [142] reported that the initial reactions of the consumers upon learning about in vitro meat were underpinned by feelings of disgust and considerations of unnaturalness. Siegrist et al. [143] reported a low level of acceptance for cultured meat due to perceived unnaturalness and disgust and pointed out that the perception of the participants was highly influenced by the description of the product. Rather than giving more importance to the production method, the product needs to be highlighted and labeled and introduced in a nontechnical way highlighting the similarities of cultured meat with conventional meat. Content-based information can play a significant role and influence the attitude of the people for the commercial success of cultured meat [144].

Although information about the sustainability and a positively perceived sustainable product could contribute to commercial success and to the acceptance of cultured meat [144], how customers see this new commodity in relation to conventional meat is mainly going to decide its commercial success [145]. Educated consumers (scientists and students) believe that cultured meat is not going to be accepted by the consumers and it is not a solution for the problems associated with the current meat production [146]. Given the conditions that prices were equal, and burgers tasted the same, only 11% of the participants opted to purchase the cultured meat–based burger in comparison to a conventional beef burger [147]. The demand for cultured meat was also reported to be price sensitive as 6% increase was seen in the share of regular beef with 1$ increase in the price of cultured meat–based burger (base price of 4$), emphasizing that the benefits of the production system might be insufficient for increasing acceptance of the consumers due to lack of naturalness associated with it [148]. This unnaturalness perceived by the consumers could be reduced by small-scale production methods that can help in reversing the feelings of alienation by allowing close contact with cell-donor animals [149]. Novel food technologies may face a lack of acceptance even if the risks are lower and benefits are higher [150,151].

Role of media in publicity of cultured meat

Media has played a great role in publicity of this new product and has been mostly projecting it as a potential solution to several problems associated with conventional meat production. While studying the media coverage of cultured meat, Goodwin and Shoulders [152] reported that several sources, mostly advocates of cultured meat, have covered many aspects of this new product such as benefits, history, process, and time and particularly highlight the problems associated with current livestock agriculture. While studying the media coverage of the tasting event of cultured meat burger in 2013, Hopkins [153] reported that there has been an over emphasis on the coverage and tenure of the coverage, particularly in Canada, the United States, and the United Kingdom. By over emphasizing the acceptance of cultured meat among vegetarians and highlighting its potential in mitigation of the environmental and animal welfare issues associated with conventional livestock agriculture, the Western media neglects the technological challenges and gaps in the knowledge that are in the way of commercial success and acceptance of this novel product [153].

Market for cultured meat

While media has given enough publicity and there has been an increased interest in cultured meat production, some of the recent studies have presented mixed results regarding the expected market size for cultured meat. Despite of the facts that an overall positive view of the consumers was reported about the cultured meat [145] and 91% of the participants agreed to the idea of giving a try to this new product in a study carried out by Verbeke et al. [154], there are some less favorable results reported by several studies such as Verbeke et al. [142], Siegrist and Sütterlin [148], Hocquette et al. [146], and Verbeke et al. [154] about the potential market for the cultured meat. However, new information through marketing campaigns or social norms have an influence and could

TABLE 73.1 Acceptance and perception of consumers toward cultured meat.

Authors	Aspect studied	Findings
Slade [147]	A hypothetical choice experiment was performed to study the consumer preference for burgers based on cultured meat	Given the conditions that prices were equal and burgers tasted the same, 65% of the consumers opted to purchase the regular beef burger and only 11% consumers chose to purchase the cultured meat burger
Siegrist et al. [143]	How does the perceived naturalness and evoked disgust affect the acceptance of cultured meat	A product-oriented approach focused on the merits of the product and explained in a nontechnical way will be more effective for the acceptance of cultured meat
Bryant and Barnett [155]	A systematic review of the available literature was done to elucidate the acceptance of consumers toward cultured meat	The authors studied various factors that affect the acceptance of cultured meat and its demographic variations, consumer objections, perceived benefits and areas of uncertainty. The study concluded that consumers perceive the environmental benefits and animal welfare advantages; however, these are only going to have minor influence on their buying decisions
Bekker et al. [144]	How does the information provision on implicit and the explicit attitude influence consumer acceptance toward cultured meat	The explicit attitude toward cultured meat can be influenced by the information about the sustainability of this production system and a positively perceived sustainable product
Siegrist and Sütterlin [148]	How does the perceived naturalness affect the acceptance of cultured meat	Despite the fact that this novel production system seems more environmental-friendly and humane, lack of naturalness perceived by consumers might reduce the acceptability of cultured meat
Verbeke et al. [142]	Reactions toward cultured meat and attitude formation of the consumers from Portugal, the United Kingdom, and Belgium was studied	Initial reactions of the consumers toward cultured meat were underpinned by the feelings of disgust and considerations of unnaturalness leading to some kind of fear of the unknown. The consumers acknowledged the global and personal merits of this production system
Verbeke et al. [154]	The authors studied the prospects and challenges of cultured meat in terms of consumer acceptance	Sensory characteristics and price of the product were identified as the major factors to influence the acceptance of cultured meat
Hocquette et al. [146]	The study mainly involved students and scientists and evaluated what educated consumers think about cultured meat	While only a minority (5%–11%) of consumers accepted to eat or recommend cultured meat, majority of consumers believed that cultured meat would not be tasty and healthy and will not solve the problems associated with current production systems
Welin [156]	The authors studied the prospects and problems associated with cultured meat	The perceived "unnaturalness" was identified as the biggest barrier for acceptance of consumers toward cultured meat

change the consumer preferences and affect the market size [144].

Conclusion

Cultured meat is promoted as a humane and clean production system offering several advantages, including animal welfare, environmental considerations, process monitoring, efficiency of food production, and reduced resource use. The composition, nutritional value, and functional role of cultured meat seem to be more manipulatable. This system holds great promises as an alternative to conventionally produced meat provided consumer resistance can be overcome. There are several technological

challenges in the path of success of cultured meat and a great deal of research is still required to fill the gaps in our knowledge. Production on an industrial scale would be feasible only when a cost-effective process creating a product qualitatively competitive with existing meat products is established. Government subsidization like that provided to other agribusinesses can play a role during initial establishment of this industry.

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Part Twenty one

Emerging technologies

Chapter 74

Three-dimensional bioprinting for tissue engineering

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Introduction

Tissue engineering strategy aims to develop biological substitutes to overcome the high shortage of autologous tissues and organs for transplantation. Although tissue engineering applications have progressed rapidly over the past two decades, the conventional fabrication methods are limited in their abilities to create clinically applicable tissue constructs with well-interconnected pores, complex architectures, patient-specific geometries, and heterogeneous material distributions. Over the past few years, three-dimensional (3D) bioprinting strategy has been applied to address these limitations [1–4]. It allows for the fabrication of tissue constructs with unique spatial control over the deposition of cells, biomaterials, and bioactive molecules, resulting in higher regenerative capability after implantation [5,6]. 3D printing or additive manufacturing was developed in the 1980s, and it included various approaches to create objects from a computer-generated file [7]. This technology quickly became a powerful tool in tissue engineering and biomedical research [8]. In the structure-based bioprinting, bioinert or bioactive materials such as metals, ceramics, and polymers are used to develop a tissue structure followed by precisely depositing cells and bioactive molecules to it [9,10]. In the cell-based bioprinting, high density of cells is patterned spatially with a prescribed organization in a layer-by-layer fashion forming tissue-like constructs [3,9].

Thus 3D bioprinting allows the creation of tissue-specific architectures with precise geometries that have been limited to conventional fabrication methods. Landers et al. first introduced 3D bioprinting as an extrusion-based method to continuously dispense cells within a hydrogel material (the bioink) from a dispensing head to a stage based on patterns predesigned through computer-aided design/manufacturing (CAD/CAM) tools [11,12]. Various types of 3D bioprinting methodologies are now available to meet the specific requirement in tissue engineering applications.

3D Bioprinting strategy: from medical image to printed bioengineered tissue

3D bioprinting strategy aims to achieve reproducible, complex tissue structures that are well vascularized and suitable for future clinical use. Since human tissues and organs have arbitrary 3D shapes composed of multiple cell types and extracellular matrix (ECM) with the functional organization, this strategy can be the most effective way to achieve this goal [1]. The CAD/CAM processes are important technologies needed for the future clinical applications of 3D bioprinting because these processes provide an automated way to replicate a 3D shape of a targeted tissue structure [13]. In general, the process starts by scanning the patient to produce 3D volumetric information of a target object using medical imaging modalities such as computed tomography (CT) and magnetic resonance imaging. These imaging tools acquire information from cross-sectional slices of the body, and the data is stored in the Digital Imaging and Communications in Medicine (DICOM) format that is a standard format for digital imaging in medicine. This information is transformed into a 3D CAD model by the reverse engineering process. This process starts with interpolation of points within and between image slices to improve resolution and generate voxels from the measured data. This CAD model is created by the extraction of localized volumetric data from a targeted tissue structure to generate a surface model. In this step the sophisticated reconstruction of the CAD model is required for bioprinting process due to a complexity of tissue or organ. A motion program, which

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is instructional computer codes for the printer to follow designed paths, is generated with a CAM system. This CAM process is divided into three steps: slicing, tool path, and motion program generation. Slicing is to obtain information of sliced two-dimensional (2D) shapes of an object for the layer-by-layer process. Then tool path generation is for creating a path for the tool to follow to fill the cross-sectional space of each layer. The printed tissue-specific architecture has the proper inner functional structure constructed with multiple cellular components for efficient tissue regeneration. Therefore a well-organized strategy for tool path generation is required. Fig. 74.1 shows 3D bioprinting strategy from the medical image to the printed tissue constructs developed by CAD/CAM process and automated printing of 3D shape imitating target tissue or organ [1,13].

Three-dimensional bioprinting techniques

A variety of 3D printing techniques has been developed to bioengineer 3D human tissue/organ constructs for tissue engineering applications. The effectiveness of each printing technique relies heavily on biomaterials choice and targeted applications. In general, bioprinters consist of three main components: three-axis stage, printing cartridges, and the dispenser. Stage controllers move the printer head in the *X*, *Y*, and *Z* directions. Printing cartridges, usually in the form of a syringe, store either the polymeric components of the scaffold or the cell-laden hydrogel components, and they include a nozzle that determines the amount of material dispensed at set printing parameters. The dispenser system is the final component, which causes the deposition of materials, and it varies between the printing techniques (Fig. 74.2).

Jetting-based bioprinting

Boland et al. demonstrated the deposition of living cells using modified commercial thermal inkjet printer [20]. Similar to the inkjet printer used to apply ink onto a paper, the jetting-based bioprinters dispense a controlled volume of liquid to a predefined location through non-contact deposition (Fig. 74.2A). Selected hydrogel acts as an "ink" in this case where it can be dispensed in volumes between 10 and 150 pL depending on the dispensing modules used [21]. The two common dispensing methods to generate droplets in inkjet printers are thermal and piezoelectric actuators. Thermal inkjet bioprinters use a thermal actuator that locally heats up the bioink to generate small bubbles in the printhead. These bubbles collapse to create pressure pulses that force droplets of liquid out of the nozzle. Although thermal inkjets use heat around 200°C−300°C, the duration of heating is typically around 2 μs which studies have found to result in only a 4°C−10°C rise in hydrogel temperature [4]. Piezoelectric inkjet bioprinters use a polycrystalline piezoelectric actuator that changes its shape when a voltage is applied. The change in the volume of the printhead induces a pressure pulse that ejects the droplet of bioink [21]. The volume of the dispensed bioink is dependent on various factors such as the temperature gradient of the printhead, the frequency of the pressure pulse, and the viscosity of the ink itself. Finally, *X* and *Y* position of

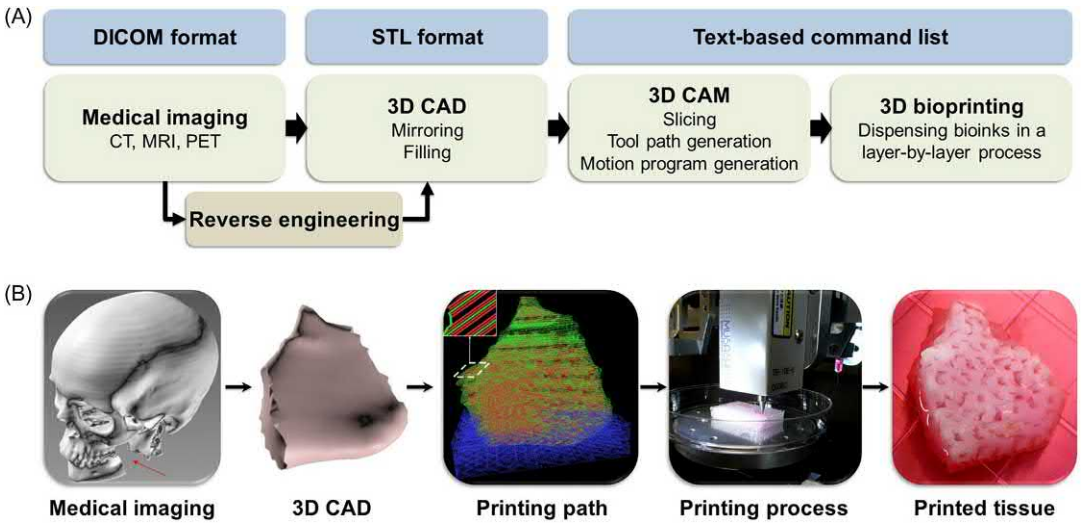

FIGURE 74.1 3D bioprinting strategy from medical imaging to printed tissue construct: (A) schematic diagram and (B) example of a CAD/CAM process for automated printing of 3D shape imitating target tissue or organ. A 3D CAD model developed from medical image data generates a visualized motion program, which includes instructions for *XYZ* stage movements and actuating pneumatic pressure to achieve 3D bioprinting. *3D*, Three-dimensional; *CAD/CAM*, computer-aided design/manufacturing.

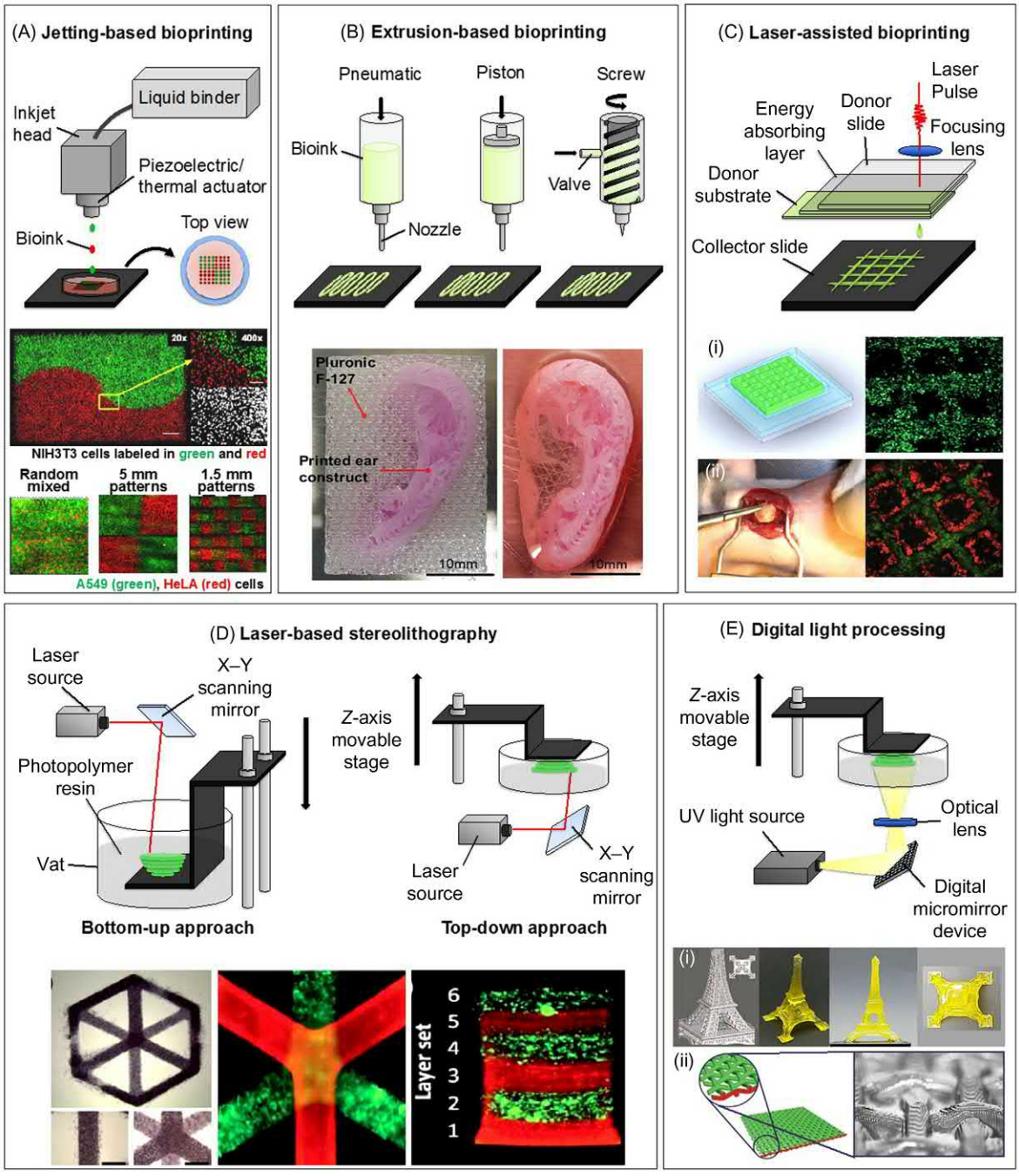

FIGURE 74.2 Schematic diagrams and examples of 3D bioprinting technologies. (A) Inkjet-based bioprinting: NIH/3T3 cells were labeled with green and red fluorescent dyes and patterned (above) [14]. A549 (green) and HeLa (red) cells were patterned in checkerboard designs with different sizes of inner squares using direct inkjet printing (bottom). (B) Extrusion-based bioprinting: auricular implant was printed with cell-laden hydrogel, PCL, and Pluronic F-127 through an extrusion-based method using pneumatic dispenser [1]. (C) Laser-assisted bioprinting: human adipose-derived stem cells-laden 3D graft (C-i) [15] and cardiac patch with HUVECs and hMSCs (C-ii) [16] were printed with laser-assisted techniques. (D) SLA: spatial 3D multilayer cell patterning of fibroblasts in PEGDA hydrogels were printed using laser-based SLA [17]. (E) DLP: a miniature of Eifel tower printed with methacrylated silk fibroin hydrogel (E-i) [18] and woven chain mail structure printed with PVAMA/GelMA bioink (E-ii) [19] using DLP bioprinter. *3D*, Three-dimensional; *DLP*, digital light processing; *GelMA*, gelatin methacrylate; *HUVECs*, human umbilical vein ECs; *EC*, endothelial cells; *PVAMA*, poly(vinyl alcohol) methacrylate; *PEGDA*, poly(ethylene glycol) diacrylate; *SLA*, stereolithography.

the nozzle head can be precisely controlled in a scale of micrometers to allow high-resolution bioprinting.

Jetting-based bioprinting offers many advantages. First, the patterning of various designs with different cell types is possible. For instance, Park et al. used a direct inkjet printing technique to directly pattern living cells (Fig. 74.2A) [14]. Initially, they printed the NIH/3T3 cells labeled with green and red fluorescent dyes to show the feasibility of heterotypic cell patterning. Then they labeled the A549 and HeLa cells with green and red fluorescent dyes, respectively, and patterned checkerboard shape with different sizes of inner squares. Inkjet printers also offer a high printing resolution where concentration gradients of cells, biomaterials, or bioactive molecules throughout the structure can be controlled by altering drop sizes and densities [4]. For instance, droplets can be arranged into linear patterns with 50 μm intervals or single droplet patterns containing only one or two cells [22]. In addition, droplet size can be controlled electronically to range from less than 1 to over 300 pL in volume with deposition rates from 1 to 10,000 droplets per second [23]. However, major drawbacks of jetting-based printing are its limited performance of 3D tissue construction. Unless cured rapidly, it is difficult to build up the 3D structure along the *Z*-axis with the jetting-based bioprinting techniques, and only low viscosity materials ($\sim$0.1 Pa/s) can be used [24]. Also, as cells are forced through the nozzle, shear stress induced during the printing process has a huge risk of damaging the cell membranes that can cause cell lysis [21]. Lastly, high cell concentrations (more than 10 million cells/mL) often result in nozzle clogging, and some instances may even alter the properties of the hydrogel greatly that it can hinder the proper cross-linking of the bioink [25,26].

Extrusion-based bioprinting

The most well-known and commercial 3D bioprinters are extrusion-based printers. Unlike jetting-based printing, extrusion-based printing uses an additive manufacturing mechanism that relies on fused deposition modeling, and the biomaterial is dispensed directly on the stage in a continuous string form rather than liquid droplets (Fig. 74.2B). The printer extrudes a 2D pattern designed by CAD/CAM software, and then each layer serves as the foundation for the next layer above it. These extrusion type printers mainly include a stage, a dispensing system, and a printing cartridge that are capable of movement in the *X*, *Y*, and *Z* axes. Three dispensing systems are widely utilized to extrude the biomaterial in the barrel for 3D bioprinting: (1) pneumatic-, (2) piston-, and (3) screw-based dispensing systems [4]. The pneumatic-based dispensing system allows accurate control over the air pressure to allow dispensing of the material with increased pressure need for more viscous materials [27]. The ultimate force of pneumatic systems is only limited by the air pressure capabilities of the system. On the other hand, mechanical dispensing systems use motor-derived piston or screw to provide more spatial control at the cost of reduced maximum force capabilities [4]. However, the rotating screw gear requires fine design to be used for bioprinting. Despite its advantages in dispensing high viscose material, the configuration of a screw can induce a high-pressure drop along the nozzle, which can potentially harm the loaded cells [9].

The main advantage of extrusion-based bioprinting is that can build 3D tissue architectures that can be implanted in vivo. For instance, Kang et al. used extrusion-based 3D printing for ear cartilage reconstruction (Fig. 74.2B) [1]. CT image of an ear was used for printing of the auricular implant with chondrocyte-laden hydrogel bioink, poly (ε-caprolactone) (PCL), and Pluronic F-127. In addition, the extrusion-based technique can use a wide range of biomaterials, including hydrogels, polymers, cell aggregates, and ceramics. Materials with viscosities ranging from 30 to greater than 6×10^7 mPa/s have all been successfully printed with extrusion-based printers providing a wide range of selection of biomaterials [28]. Another advantage of extrusion-based printing method is its ability to print bioink containing a high density of cells. However, one of the downsides is that an increase in pressure and nozzle gauge can induce high shear stress causing a decrease in the viability of cells. Therefore the size of the nozzle and level of pressure should be carefully selected to ensure high cell viability after printing. Another disadvantage of extrusion-based printing is its limitation in printing resolution and speed relative to other bioprinting techniques. For instance, nonbiological extrusion printers can print 5−200 μm resolution at linear speeds of 10−50 μm/s [29]. Using biomaterials and cells, the minimum resolution is generally over 100 μm which is lower than the resolution in other bioprinting methods [30].

Laser-assisted bioprinting

Laser-assisted bioprinting is a 3D printing method to shape and assemble bioink by laser-guidance direct writing [31]. In 1999 Odde et al. performed the direct writing of living cells (embryonic and chick spinal cord cells) [32]. It is operated by focusing a laser pulse toward an absorbing layer, typically gold or titanium, to generate high-pressure bubbles that propel cell-containing bioink toward a collector slide [4]. A standard laser-assisted printing system usually consists of a pulsed laser beam, a focusing lens, a donor slide, a "ribbon" that has transport support made from a laser-energy-absorbing layer, a donor substrate that has hydrogel or cellular material layer and a collector slide (Fig. 74.2C). The laser-assisted methods can deposit materials with viscosities ranging from

1 to 300 mPa/s and cell densities close to 10^8 cells/mL with resolutions close to a single cell per drop without significant effects on cell viability or function. There are many factors that affect printing resolution. Some of these factors include the energy delivered per unit area due to the laser, the surface tension, substrate wettability, the gap between the donor substrate and collector slide, and the thickness and viscosity of the biological layer [4].

Similar to jetting-based bioprinting, one of the reasons for using the laser-assisted technique is its ability to pattern cells in various designs. For instance, Guillotin et al. used alginate-based bioink with human endothelial cells (ECs) to print patterns of the Olympic rings and two concentric circles using laser-assisted bioprinting [33]. Similarly, Gaebel et al. printed cardiac patch made of human umbilical vein ECs (HUVECs) and human mesenchymal stem cells (hMSCs) using laser-induced forward transfer (LIFT) cell printing technique (Fig. 74.2C-i) [16]. Furthermore, Gruene et al. developed 3D tissue graft made of human adipose−derived stem cells (ASCs)−laden alginate hydrogel (Fig. 74.2C-ii) [15]. Another advantage of laser-assisted bioprinting method is that it is nozzle free. Therefore nozzle clogging issues often seen in jetting-based and extrusion-based bioprinting systems are avoided. However, there are also several disadvantages associated with the laser-assisted printing system. The major concern is its potential risk of damaging the cells caused by the thermal energy of the laser [33]. It can also be difficult to accurately target and position the cells due to the nature of the ribbon cell coating. Moreover, metallic residues may be present in the final construct due to the vaporization of the laser-absorbing layer. To reduce this contamination, there have been methods of using nonmetallic absorbing layers and altering the printing process so that an absorbing layer is not needed [34].

Laser-based stereolithography

On August 8, 1984, stereolithography (SLA) 3D printer was developed by Chuck Hull, which was the world's first 3D printer [35]. The SLA printing technique creates 3D objects by successively photo-curing thin layers of resin by a spatially controlled laser beam. The main components of a laser-based SLA 3D printer are a laser source, an X−Y scanning mirror, a vat (resin tank), photo-curable polymer resin, and a movable stage (Fig. 74.2D) [36]. Depending on the direction of fabrication, the printer has two approaches: bottom-up and top-down methods. Early developed SLA printers used the bottom-up approach where the printing stage lowers after the curing of the first layer to cover the topmost layer with unpolymerized resin. Another layer of resin is sequentially cured and the stage lowers again. The critical limitation of the bottom-up approach is that it requires a large vat of resin, which causes enormous consumption of materials and high expenditure. On the other hand, for the top-down approach, the light source is projected through a transparent film on the bottom of the vat filled with photo-polymers. The first layer is cured with light and is detached from the transparent film as the printing stage moves upward. Another layer of uncured photo-polymer fills in the gap between the first layer and the bottom of the vat. The second layer is printed on the bottom surface of the previous layer, and this process is repeated until the printing is over. The unpolymerized resin is removed by draining. The top-down approach uses a much smaller resin tank allowing a lower cost of material preparation. Due to this characteristic, many bioprinting studies with SLA technique use top-down approaches.

Digital light processing

Digital light processing (DLP) is another type of SLA. The major difference of DLP from laser-based SLA is that DLP uses a projection of ultraviolet (UV) light (or visible light) from a digital projector to flash a single image of the layer across the entire resin at once. One of the key components of DLP is a digital micromirror device (DMD) chip (Fig. 74.2E). DMD chip is composed of an array of reflective aluminum micromirrors that redirect incoming light from the UV source to project an image of a designed pattern or a layer of a 3D CAD model. The projection of an image that is composed of small square pixels called "voxels" passes through an optical lens and cures the photo-curable polymer resin [37]. To fabricate a high-resolution structure, setting up the parameters such as the curing time of each layer, layer thickness, and intensity of the UV light is critical. These parameters are highly dependent on the concentration and types of photopolymer and photo-initiator used for the resin.

There are many advantages of laser- and DLP-based SLA bioprinting techniques over extrusion-based methods. The SLA bioprinter has the ability to fabricate a complex 3D model with high resolution ($\sim$1.2 μm) [38]. Another advantage of SLA process is to precisely control the average energy dose to minimize the adverse effect on cells [17]. Chan et al. used SLA printer with both modified top-down and bottom-up methods to fabricate fibroblast-encapsulated 3D multilayer structure (Fig. 74.2D) [17]. The DLP bioprinter also can print an intricate structure with high resolution ($\sim$1 μm) and with fast printing speed ($\sim$30 min, mm^3/s) regardless of the layer's area and pattern [18]. Kim et al. printed a miniature of Eifel Tower with methacrylated silk fibroin bioink using DLP-based SLA printing (Fig. 74.2E-i) [18]. Moreover, the flexibility to fabricate complex 3D designs and integrate a variety of

functional elements including live cells, biomaterials, and nanoparticles can be achieved with these computer-aided, photo-curing-based techniques [39]. For instance, Lim et al. used MSCs-laden poly(vinyl alcohol) methacrylate (MA)/gelatin MA (GelMA) hydrogel to print complex tangled structure such as a woven chain mail (Fig. 74.2E-ii) [19]. Also, physical masks or molds are not needed in DLP 3D printing. However, the major downside of these SLA printing techniques is that there are limited biomaterials for the bioink that can be photo-cured [18]. Poly(ethylene glycol) (PEG) diacrylate (PEGDA), GelMA, Pluronic F127 DA, and hyaluronic acid (HA) MA are reported as potential biomaterials for DLP printing [40,41].

Hybrid and other techniques

Hybrid fabrication system is a combination of technologies that are individually practiced. Despite the rapid growth over two decades, the current 3D bioprinting techniques and systems fall short in integrating rigid and soft multifunctional components. In some instances the advantages of one technology might not be sufficient to meet the requirement of creating targeted functional tissue constructs. Merging technologies may overcome this limitation and improve the bioprinting process. The combination of technologies can be at the cellular level, bioink level, or on the bioprinter level. Tan et al. introduced a 3D bioprinting strategy by integrating conventional scaffold-based fabrication method and the cell-based bioprinting approach [42]. They explored the use of hydrogel encapsulated cell-laden microspheres as the bioink for 3D bioprinting. The hydrogel lubricates and glues the microspheres during printing and helps in fusing together after printing upon gelation. Kucukgul et al. developed computational algorithms to generate support structures of cylindrical cell aggregates for printing fully cell-based vascular structures [43]. Xu et al. proposed a combination of electrospinning and inkjet bioprinting techniques to develop layered cartilage constructs [44]. Combining the two different principles of electrospinning and bioprinting resulted in the fabrication of cartilage construct with appropriate cell function and mechanical properties. Shanjani et al. developed a new hybrid 3D bioprinting technology, called Hybprinter, by integrating soft and rigid components [45]. This technique employs DLP-based SLA component and molten material extrusion techniques. PEGDA was used as a soft hydrogel, and PCL was used for the scaffold's structural support. Mendoza-Buenrostro et al. reported a hybrid bioprinting technique for the fabrication of scaffolds with topography scales ranging from nanometers to millimeters [46]. Scaffolds were produced by extrusion printing PCL embedded with nanofibers.

Biomaterials as bioinks for three-dimensional bioprinting

Hydrogel-based bioinks for cell-based three-dimensional bioprinting

In bioprinting a hydrogel that can encapsulate and deliver tissue-specific cell types with bioactive molecules through the printing mechanisms is referred to as bioink. Generally, hydrogels are made from naturally derived or synthetically produced hydrogels that are biocompatible, biodegradable, and chemically or physically cross-linkable to maintain a user-defined structure that ultimately assists cell viability, proliferation, differentiation, and function [47]. Hydrogels are also highly moist and ideal for absorbing high levels of nutrients and oxygen, allowing cells to survive within the construct and diffuse waste. Due to these characteristics, hydrogel-based bioinks have been used in a variety of bioprinting techniques to directly deliver cells in the printed constructs.

The required properties of hydrogel-based bioinks

To ensure successful 3D bioprinting, printability of hydrogel-based bioink is critical. As the number of manufacturing techniques capable of bioprinting has increased over time, including jetting-, extrusion-, laser-assisted printings and SLA-based printing, the physical and rheological requirements for a feasible bioink vary upon printing methods [48]. Fig. 74.3 shows the schematic diagram of variables essential to 3D bioprinting strategy. Depending on the printing techniques used, hydrogels' material properties, cross-linking mechanisms, and printing parameters should all be carefully selected to improve the printability of bioinks.

In the extrusion-based bioprinting the required properties of hydrogel-based bioinks are (1) relatively sufficient viscosity to maintain homogenous cell suspension, (2) strong shear-thinning behavior to minimize cell damage, and (3) rapid cross-linking to build a 3D tissue architecture [3,49]. First, bioinks should have proper viscoelastic properties that allow them to have homogenous cell suspension and initial structural integrity to be printed in multiple layers before cross-linking. Second, bioinks should have strong shear thinning behavior, which is the non-Newtonian behavior of fluids meaning that viscosity decreases under shear strain [50]. The shear thinning behavior allows the otherwise stiff hydrogels injectable and minimizes cell damage during extrusion. Last, the rapid cross-linking of bioink is critical since most hydrogels cannot self-support upon layer-by-layer deposition [51]. Due to adverse effects of dense polymer matrices that can inhibit the remodeling and

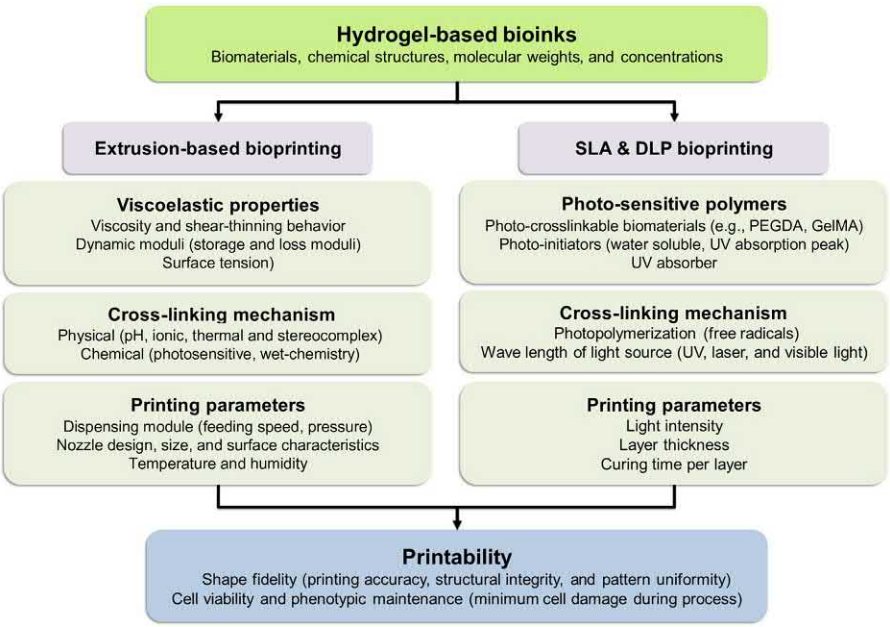

FIGURE 74.3 Schematic diagram of variables critical to 3D bioprinting strategy. The material properties, cross-linking mechanism, and printing parameters all needs to be carefully selected in hydrogel-based bioprinting. *3D*, Three-dimensional.

vascularization of cells, the traditional hydrogel precursors are low viscous solutions that are cross-linked either during or after the printing process [52]. Cross-linking before printing increases the shear stress on the cells, resulting in the high potential of cellular damage and nozzle clogging. Meanwhile, cross-linking after printing affects the resolution due to the spreading of bioink in the time between extrusion and cross-linking, which can lead to incomplete cross-linking in large multilayered constructs [4].

In the laser-based SLA and DLP bioprinting, the hydrogel-based bioinks are photopolymerized by a pattern of laser or projected UV light, respectively, to form 3D structure [18]. Therefore understanding of the photo-curing mechanism is essential. Briefly, a photo-initiator generates free radicals when absorbed with UV light (initiation). The free radicals abstract hydrogen from a double bond of polymer to generate new free radicals that abstract hydrogen from another polymer, and this process repeats to build a strong polymer network (propagation). Finally, two free radical species react with each other to create a stable, nonradical state (termination). Thus the hydrogel-based bioinks that are feasible for SLA and DLP must exhibit characteristics that are compatible with the lithographic process and which differ notably from the requirement of extrusion-based bioprinting [53]. Typically, GelMA and PEGDA are widely used photo-crosslinkable biomaterials in SLA and DLP bioprintings [54]. Also, the photo-initiator in the bioink needs to be carefully selected. The photo-initiator needs to be soluble to water, biocompatible, and have a wavelength of maximum absorbance that matches with the wavelength of light source used. Lastly, UV-absorber such as color dyes can also be added to avoid the overcuring of layers beyond the focal plane to enhance printing resolution.

Since the cell-based bioprinting aims to build a 3D tissue construct containing live cells, the cell viability after printing is one of the main criteria for printability of bioinks. For the cells to survive a biologically favorable microenvironment is required so that the cells can be well preserved not only during the printing process but also afterward in culture. In the hydrogel-based bioinks, the cell density, diffusion coefficient, temperature, and humidity can significantly affect the printability of the bioinks [55–57]. In addition, regardless of printing techniques, a sterile condition for cell printing must first be acquired to ensure high cell viability and prevent contamination after printing. The bioink and components of printer that directly contact with the cells must be sterilized before use.

Synthetic hydrogels

Synthetic hydrogels used as bioinks have low cytotoxicity, controlled biodegradability, and good mechanical properties; however, most of the synthetic hydrogels have low biological properties that minimally interact with cells. Pluronic F127, a thermosensitive hydrogel, undergoes a phase transition at room temperature and becomes a viscous substance [58]. When the concentration of Pluronic F127 is 25% w/v or more, it can be dispensed with high printability. Although high resolution of the printed structures can be achieved using Pluronic F127, the structure can be easily collapsed in the culture condition. To improve its mechanical stability, Pluronic F127 has been used chemically

modified as a photo-crosslinkable hydrogel [59]. In addition, Pluronic F127 hydrogel has been widely used as a sacrificial bioink to support the 3D architecture because it is easy to print uniformly and be immediately washed out once printing is complete [1].

PEG-based hydrogels are also widely used in 3D bioprinting because they can be chemically modified and functionalized to improve their biological and biomechanical properties by introducing various functional motifs attached to the terminal end of PEG [60] or combining with other hydrogels [41,61]. In order to improve the mechanical properties of PEG, the addition of DA of MA has been used for various applications. Thus photopolymerization of PEG-based hydrogels could achieve the tunable mechanical properties in the bioprinted constructs.

Naturally derived hydrogels

Natural hydrogels are classified into mainly proteins and polysaccharides. Most of them are present in the body, so they show high biological properties and do not cause a severe immune response. Collagen (mainly, type I) is the most abundant component of ECM, and it contains the cell-guiding chemical cues such as the cell adhesion peptide sequence, arginine−glycine−aspartic acid (RGD). Under the appropriate temperature and pH, a pure collagen solution physically forms a gel with properties dependent on the solution concentration. However, collagen itself has rarely been used as a bioink material due to low viscosity and poor mechanical stability. In order to overcome these limitations, the collagen has been mixed with various other hydrogels such as agarose, chitosan, fibrin, HA, and/or other materials [44,62−64]. On the other hand, Kim et al. determined collagen's rheological behaviors with various printing temperatures to improve the stability of the collagen-based bioinks without any additional hydrogels [65,66]. The results showed that the proper structural stability of printed collagen construct was achieved at 5°C−10°C for the printing nozzle and 35°C−37°C for the printing stage, followed by tannic acid cross-linking process. An approach for printing skin cell−containing collagen-based bioink from separate nozzles using an inkjet micro-valve dispensing method was reported [67]. In the printing process the collagen solution remained acidic and cooled. Multiple layers of cell-laden collagen bioink were printed and then treated with aerosolized sodium bicarbonate ($NaHCO_3$) to buffer the pH toward neutral for gelation. The results showed high cell viability at 1 day after printing for keratinocytes and fibroblasts, indicating the survival of cells and spatial control of the printing approach which is needed to offer a functional skin replacement.

Gelatin is a substance in which water-insoluble collagen is made soluble by high temperature or acid/base treatment. It forms a thermo-reversible hydrogel. Moreover, chemical modification of gelatin with unsaturated methyl MA results in GelMA, which can form covalently cross-linked hydrogels under UV exposure [51]. Recently, photo-crosslinkable GelMA is the most popular bioink material for cell-based bioprinting due to proper printability and tunable mechanical properties. GelMA has been used for printing a complex architecture, which contained various cell types and vasculature [68]. For instance, a cell-laden GelMA and sacrificial Pluronic F127 were dispensed and embedded within the GelMA block in predetermined 3D structure. After then, the printed structure was cross-linked by UV exposure. The sacrificial Pluronic F127 was removed at 4°C by the phase transition to create open microchannels within the GelMA block. Lastly, a suspension of HUVECs was seeded into the open microchannels. This approach allowed for the viable deposition of cells in 3D structure with a microvessel-like structure that was covered by ECs for the provision of nutrients to surrounding cells.

HA is the most abundant of the glycosaminoglycan (GAG) family, which has the repeating structure of glucuronic acid and *N*-acetyl-glucosamine disaccharide. HA has a high molecular weight and a large amount of branching that allow for intermolecular hydrogen bonding and high viscosity. Like other polysaccharides, HA can support cell survival but has low cell-binding motifs. Moreover, HA itself has very low structural integrity and shape fidelity after printing; therefore HA has been chemically modified for cross-linking or mixed with other hydrogels [69]. For example, Skardal et al. developed a bioink formulation combined with methacrylated HA and GelMA and photo-crosslinked by a two-step process, before and after extrusion, to form more compact firm constructs [61,70].

Fibrinogen, which is a glycoprotein, is reacted with thrombin to convert into fibrin network self-assembles [71]. Fibrin has many cell-binding motifs that allow the cell attachment and vulnerability to proteases for remodeling. Fibrin-based bioink has been used by LIFT-based printing process for a 3D multicellular array [72]. To improve the gel stability, HA was added to fibrinogen solution to print the arrays. Endothelial colony-forming cells (ECFCs) were printed along with ASCs in 3D patterns that a 9×9 array of ASC droplets were printed followed by an inset 8×8 array of ECFCs. These droplet arrays were printed onto a layer of fibrinogen−HA which was spray treated with thrombin−calcium chloride solution to induce the gelation. The cell-laden droplets were converted to fibrin-HA as they encountered the treated substrate with a residual thrombin solution. The results showed that ASCs initially migrated toward ECFCs without evidence of ECFC sprouting or migrating at all. Once ASCs contacted the ECFC aggregates, an explosion of ECFC network sprouts began to extend from the initial

droplet position and remained as stable networks for several weeks.

Alginate is a naturally derived anionic polysaccharide exhibiting gelation in the presence of divalent ions such as Ca^{2+} [73]. Alginate hydrogel has served as a cell delivery material for many tissue engineering and drug delivery applications due to ease of preparation and relatively good cell compatibility; however, the major drawback is the lack of mammalian enzymatic degradation, which limits tissue remodeling when implanted. In 3D bioprinting, alginate solution in 2%–4% (w/v) is extrudable and structurally stable after cross-linking process. In addition, it can be mixed with other materials to increase the printing resolution as a bioink. For example, the alginate solution mixed with cellulose nanofibers improved shear fluidization and the viscosity, resulting in high printing resolution. Then alginate was cross-linked with the divalent cation Ca^{2+} to stability the printed construct. In the early stage of cell-based bioprinting, a jetting printing setup was modified for 3D printing by printing of a Ca^{2+}solution into a reservoir of cardiac cells mixed with alginate solution [74].

Tissue-specific extracellular matrix–based hydrogels

Tissue-specific ECM–derived bioinks have been introduced for 3D bioprinting [75]. These ECM materials can either be obtained from cell-derived ECMs that are secreted during in vitro culture, or derived directly from native tissues through a decellularization process during which all the cellular components removed to avoid adverse immunological response [76–81]. To utilize the tissue-derived ECM materials as bioinks, the ECM-rich materials can be solubilized to reformulate as a gel type [82,83]. Importantly, the ECM provides a structural architecture that contains adhesion sites for cell surface receptors [84] and preserves normal tissue function by its tissue-specific mechanical and biochemical properties [85]. The interaction between cells and the surrounding ECM regulates a variety of physiological cellular processes, including motility, migration, invasion, and proliferation [86,87]. Moreover, the ECM regulates signal transduction pathways by binding to integrins or by modulating the activity of signaling molecules [88]. The ECM hydrogels are composed of the structural and functional molecules that characterize the native tissue ECM such as collagen, laminin, fibronectin, growth factors, GAGs, glycoproteins, and proteoglycans [89]. Thus bioinks derived from decellularized tissue-specific ECM can provide these same functions as naturally occurring ECM [90–92]. However, ECM-based hydrogel is inherently low viscous, exhibiting low shape fidelity and structural stability. Thus various attempts have been performed to enhance their chemical and physical properties in 3D bioprinting [93].

Biodegradable synthetic polymers for structure-based three-dimensional bioprinting

Synthetic polymers run some advantages for applications in tissue engineering and 3D bioprinting. These polymers can be synthesized with reproducible quality and fabricated into various structures with predetermined bulk/surface properties. Additional advantages include the capability to tailor the biomechanical properties and biodegradation kinetics for various biomedical applications. In bioprinting, synthetic polymers such as PCL, poly(lactide-*co*-glycolide) (PLGA) and poly(lactic acid) can provide the mechanical strength, thereby overcoming limitations of the hydrogel-based constructs on size, shape, and structural integrity [1]. For the extrusion method, melted thermoplastic polymers or polymer solutions with proper viscosity are needed. Therefore PCL is the most commonly available polymer for the extrusion-based bioprinting because of its low melting point of 60°C and high printability.

Unlike the extrusion-based bioprinting, SLA offers several benefits when compared to other printing techniques, most notably in the ability to print more complex architectures with high resolution. However, this printing technique requires photo-crosslinkable polymeric resins, which can be limiting for tissue engineering applications. A few of polymeric biomaterials has been adapted for use with SLA printers. For example, Dean et al. utilized poly (propylene fumarate) (PPF), which is photo-crosslinkable, for DLP printers [94,95]. The results showed that PPF was printed highly complex constructs with controlled chemical properties and porous architecture. Many research groups are exploring the synthesis of polymeric biomaterials for 3D bioprinting.

Scaffold-free cell printing

3D tissue structure can be printed using multicellular aggregates without supporting materials [96]. This method enables to dispense cell aggregates from a capillary to form 3D structure. Norotte et al. reported a scaffold-free cell printing method using multicellular spheroids consisting of smooth muscle cells (SMCs) and fibroblasts and agarose hydrogel for temporary support [97]. After printing the printed cell aggregates were fused and formed a small diameter vessel-like tube ranging from 0.9 to 2.5 mm in diameter. Li et al. fabricated vertical channels without the use of a supporting material by taking advantage of the gelation behavior of gelatin in combination with the cross-linking of alginate or fibrinogen [98]. Itoh et al. proposed a method to fabricate a scaffold-free vascular tube using the cell aggregates [99]. Predesigned 3D tubular structures were constructed with the cell aggregates consisting of ECs, SMCs, and fibroblasts. The multicellular aggregates self-organized and fused into a

tubular structure that was perfused with a bioreactor. In order to avoid the formation of droplets at the end of the nozzle due to surface tension, Ozler et al. developed a quantitative model to predict the success of cell aggregate extrusion [96]. This approach can be repeated for different cell types after obtaining their respective rheological properties. In addition, cell viability can be dependent on the compression ratio applied during the printing process. Thus it is necessary to first investigate the impact of compression on their survival rate and cellular functions.

Three-dimensional bioprinting in tissue engineering applications

Three-dimensional bioprinted vascular structures

A major limitation in the bioengineered tissue constructs is the lack of proper vascularization into the implanted constructs. Importantly, fully vascularized tissue constructs are required to attain long-term cell survival and tissue functions. 3D tissue constructs packed with metabolically active cells can rapidly be formed necrotic cores in the absence of a vascular network due to a limitation of the transport of nutrients and other physiologically relevant molecules toward and away from the tissue [100–102]. When a 3D tissue construct with cells is implanted, efficient mass transfer requires intact microvasculature to maintain the metabolic functions of cells deep inside the construct. Indeed, the ingrowth of the microvascular system into the implanted bioengineered tissue constructs in a timely manner is the key to success in clinical use [4]. Hence, many attempts have utilized 3D bioprinting as a promising technology to fabricate vascularized tissue constructs (Fig. 74.4) [68,97,103–105].

One of the earliest and simplest approaches to fabricate a vascularized tissue construct is to use a sacrificial component that acts as a structural role during printing and is later removed to create a hollow tunnel. For example, Miller et al. reported a vascular-casting approach with carbohydrate glass as the sacrificial template (Fig. 74.4A) [103]. They dispensed a cell-laden hydrogel into a mold with the lattice structure of carbohydrate glass which was removed after cross-linking of the hydrogel. Then HUVECs were injected into the generated lumen to form vascular networks. On the other hand, extrusion-based bioprinting technique has been also used to form a vascular construct. For instance, Kolesky et al. showed a method of fabricating 3D constructs completed with vasculature, including multiple cell types and ECM proteins (Fig. 74.4B) [68]. Coprinting two bioinks of HUVEC (red) and human neonatal dermal fibroblast cells (green)–laden GelMA formed 3D microvascular networks.

Although bioprinting technologies have evolved to a level of creating complex vascularized constructs carrying multiple cell types and ECM proteins, the reconnecting of the vascular structures to host circulatory system is still challenging. To address this, Lee et al. developed a novel bioprinting approach to creating a network from capillaries to large perfused vascular channels (Fig. 74.4C) [104]. The large channels were fabricated using 3D bioprinting and angiogenic sprouting of ECs from the edge of the large vessel was achieved through a natural maturation process. These printed constructs are often thin or hollow structures, so they are nourished by diffusion from host vasculature but not by anastomosing [106]. When the diffusion limit needed by engineered tissues exceeds 150–200 μm, a precise vascular network must be embedded into fabricated constructs, a feat that has not yet been accomplished [107,108]. Efforts to simplify the complex fabrication methods and to find new technologies for the bioprinting of vascular structures are currently in great need by tissue engineering as a whole.

There are also other vascularization techniques such as scaffold-free engineered tissue constructs and 4D bioprinting. First, the scaffold-free bioprinting avoids the limitation related to the foreign materials and exogenous scaffolds such as adverse host responses and infection [99]. Norotte et al. developed macrovascular tubular structures from multicellular cylinders using scaffold-free bioprinting (Fig. 74.4D) [97]. Double-layered vascular tubes similar to vessels were developed with human umbilical vein SMCs (green) and human skin fibroblasts (red), and H&E staining revealed a distinct boundary between the SMCs and fibroblast layers after 3 days of fusion. Another vascularization technique is a 4D bioprinting of self-folding tubes developed by Kirillova et al. (Fig. 74.4E) [105]. Thin films of methacrylated alginate and HA were printed and cross-linked with green visible light. Then the hydrogel films exhibited instant self-folding into tubes when immersed in water, phosphate-buffered saline (PBS), or cell culture media. The 4D bioprinting methods may allow the future development of reconfigurable tissue structure with controllable functionality and reactivity.

In vitro tissue models

Drug discovery is an inefficient process with a high failure rate and an extreme financial burden. Animal studies are not always indicative of the results in human trials, and the regulatory environment is becoming stricter as time progresses. In addition, from a moral standpoint, attempts should be made to reduce the number of animal studies conducted. In vitro studies, mostly 2D cell culture methods, are also severely limited. Drug response, gene expression, migration, morphology, and viability of cells

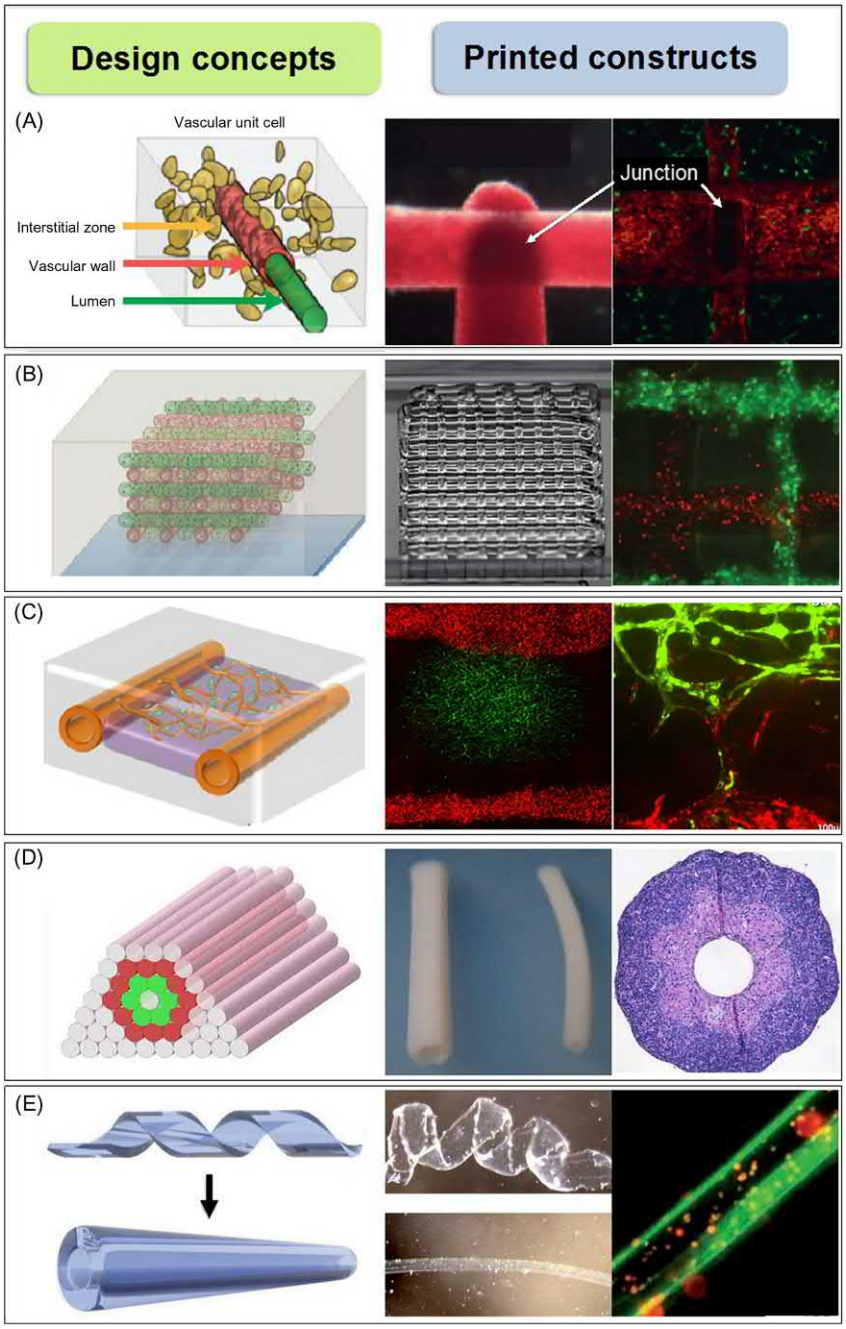

FIGURE 74.4 Examples of the 3D bioprinted vasculature. (A) The vascular unit printed by Miller et al. [103]. The construct shows three compartments consisting of the vascular lumen, EC lining, and the matrix encapsulated cells. Endothelialized channel walls and the intervessel junction surrounded by human fibroblasts. (B) The engineered tissue construct developed by Kolesky et al. [68]. Multilayers of channels lined with HUVEC (red) and HNDFs (green) formed 3D microvascular networks. (C) 3D bioprinted multiscale vascular systems performed by Lee et al. [104]. Fibrin-HUVEC cell mixture (green) was deposited in between the two vascular channels (red), and the integration of capillary network was observed at the border. (D) Scaffold-free, macrovascular tubular structures from multicellular cylinders by Norotte et al. [97]. Double-layered vascular tubes similar to vessels were developed with HUVSMCs (green) and HSFs (red), and H&E staining showed the distinct boundary between the smooth muscle cells and fibroblast layers after 3 days of fusion. (E) 4D biofabrication of self-folding tubes developed by Kirillova et al. [105]. Green visible light cross-linked methacrylated alginate and methacrylated HA hydrogel films exhibited instant self-folding into tubes when immersed in water, PBS, or cell culture media. *3D*, Three-dimensional; *EC*, endothelial cells; *HUVECs*, human umbilical vein ECs; *HA*, hyaluronic acid; *HNDFs*, human neonatal dermal fibroblast cells; *HUVSMCs*, human umbilical vein smooth muscle cells; *HSFs*, human skin fibroblasts.

have all been shown to differ between 2D and 3D environments. Recently, a large emphasis has been placed on creating in vitro 3D tissue models to overcome these limitations. Typically, this is done by suspending cells or organoids (or cell aggregates) in a 3D culture within a singular or entire array of microfluidic devices. Already, 3D in vitro assay systems have advanced immensely to a level in which living constructs can closely mimic the native tissue environment in a high-throughput platform. While several fabrication techniques have been used to develop these models, 3D bioprinting technologies are advantageous due to their low cost and efficiency, high throughput, excellent reproducibility, and the ability to create complex geometries. The major two areas to which 3D printed in vitro tissue models have been applied are cancer research and drug screening systems (Table 74.1).

Tumor models

3D bioprinting of cells as tumor models are helpful to study the interaction of immune and tumor cells and for the screening of new treatments [118]. Xu et al.

TABLE 74.1 Applications of bioprinting in vitro biological systems.

Tissue/ organ	Bioprinting method	Cell type	Encapsulation material	Testing	Outcomes	References
Tumor/ cancer	Pneumatic cell droplet patterning	Fibroblasts and ovarian cancer cells	Matrigel	Reproducibility and precision of cell density and spacing	Bioprinting methods showed improved performance compared to micropipette ejection	[109]
Tumor/ cancer	Temperature controlled, pneumatic extrusion	Hepatic carcinoma and mammary epithelial cells	Matrigel	Radiation shielding capabilities of the prodrug amifostine	Amifostine provided radioprotection to the cells, with the greatest benefit seen in the dual-cell model	[110]
Tumor/ cancer	Projection stereolithography (UV exposure)	HeLa and 10T1/2	PEGDA	The effect of channel width on tumor cell and 10 T1/2 migration and morphology	Tumor cells showed increased migration speed and less change in morphology with smaller channel sizes compared to 10 T1/2	[111]
Tumor/ cancer	Extrusion	HeLa	Gelatin–alginate–fibrinogen	Viability, proliferation, MMP expression, and chemoresistance versus 2D culture	3D cell culture increases cancer cell proliferation, MMP expression, and resistance to chemotherapy	[57]
Tumor/ cancer	Continuous 3D projection (with nonlinear exposure)	Breast cancer cells	PEG	Long-term culture and validation of breast cancer spheroids	Spheroids showed hypoxic cores and signs of necrosis, key features of tumor environment	[112]
Liver	Extrusion	Hepatocytes	Alginate	Validation of liver cell activity and metabolic performance	Cells were viable, proliferative, synthesized urea, and metabolized EFC to HFC	[113,114]
Bacterial infection	Inkjet	*Escherichia coli*	Alginate	Treatment of the *E. coli* with several common antibiotics	Similar results to the current low throughput, less reproducible, and more expensive methodologies	[115]
Brain	Extrusion and subsequent dissolution of sacrificial resin	Mouse brain ECs	Type 1 collagen microchannels	Model validation with transendothelial permeability measurements and hyperosmotic mannitol disruption test	Permeability decreased over 3 weeks of culture, and then recovered over 4 days after hyperosmotic mannitol disruption	[116]
Lung	Extrusion	ECs and epithelial cells	Matrigel	Cell viability, distribution, morphology, and permeability compared to manually placed cells	Printed constructs resulted in more homogenous cell distributions, proper cell morphologies, lower permeability, and similar viability	[117]

2D, Two-dimensional; 3D, three-dimensional; ECs, endothelial cells; EFC: 7-ethoxy-4-trifluoromethylcoumarin; HFC, 7-hydroxy-4-trifluoromethylcoumarin; MMP, matrix metalloproteinase; PEGDA, poly (ethylene glyocol) diacrylate; PEG, poly(ethylene glycol); UV, ultraviolet.

introduced a 3D in vitro cancer model using human fibroblasts and ovarian cancer cells on a Matrigel matrix, showing precise and reproducible control over cell density and spacing compared to manual ejection by micropipettes [109]. Snyder et al. also introduced a similar model in 2011 with human hepatic carcinoma cells and mammary epithelial cells. Cells and the microfluidic device were printed in order to test the radiation shielding of the prodrug amifostine. Radioprotective benefits for the liver were seen in the in vitro model [110]. Huang et al. examined tumor cell migration in a honeycomb structure with different channel widths (25, 45, and 120 μm) to mirror that of natural blood vessels [111]. HeLa and 10T1/2 cells were seeded within the device and were evaluated in the different channel sizes. HeLa cancer cells showed less morphological changes between channel sizes than 10T1/2 cells and also migrated at higher rates as channel size decreased. Also looking at bioprinted HeLa cells, Zhao et al. examined cell response after extrusion in a gelatin–alginate–fibrinogen hydrogel in comparison to a 2D culture model. Cells in the 3D model showed higher proliferation, matrix metalloproteinase expression, and chemoresistance [57]. Hribar et al. have used 3D projection printing to create concave PEG structures that form and maintain breast cancer spheroids for long-term culture [112]. The breast cancer spheroids exhibited necrotic, hypoxic cores that are key components of the tumor in vivo microenvironment.

Tissue-specific models

The number of in vitro drug screening systems has increased immensely both in quality and in quantity over the last decade, ranging from hepatic cells suspended in a microfluidic device to integrated, multiple-tissue, body-on-a-chip systems. Chang et al. used direct cell writing bioprinting to create a 3D micro-organ housed using soft lithographic micropatterning [113]. Alginate-encapsulated hepatocytes printed in the microfluidic device were viable, proliferated, and capable of synthesizing urea. This work was further investigated by infusing the hepatocyte containing a microfluidic device with 7-ethoxy-4-trifluoromethylcoumarin that was metabolized into 7-hydroxy-4-trifluoromethylcoumarin, mimicking the in vivo behavior of the liver [114]. Rodriguez-Devora et al. developed an inexpensive drug-screening platform via inkjet bioprinting deposition. *Escherichia coli* was printed in an alginate solution with different antibiotic droplets patterned on the cells, resulting in similar bacteria inhibition when compared to the current screening process [115]. An in vitro model of the blood–brain barrier has been developed by Kim et al. Mouse brain ECs were cultured within an array of type I collagen microchannels fabricated using microneedles on a 3D printed frame [116]. The model was validated by measurements of transendothelial permeability and a disruption experiment by hyperosmotic mannitol. In addition to the blood–brain barrier, an air–blood barrier has also been developed to model the lung. Horvath et al. developed a model by bioprinting epithelial cells and ECs separated by a basal membrane layer [117]. The bioprinted model was more reproducible and had thinner cell layers than which could be manufactured using traditional manual methods.

In addition to these already successful in vitro models, several areas for future work stand out in this new field. Work is in progress to incorporate an array of tissue types into the same drug-screening platform. Bioprinted in vitro models are also in good position to evaluate gene therapy techniques; however, standardized model systems/industry standards are needed to facilitate comparison across studies. The use of these in vitro models shows promise in increasing our understanding of biology, disease progression, organ cross talk, and many other areas as the field progress forward.

Three-dimensional bioprinted implantable tissue constructs

3D bioprinting has been utilized to fabricate constructs targeting nearly every tissue type in the body. While clinical implantation is still rare for this relatively new technology, there have been many successes in vitro and in vivo. Highly detailed, anatomically correct, and patient-specific tissue constructs have been fabricated for a number of tissues and organs (Table 74.2). A wide range of cells has been shown to maintain their viability, gene expression, and functional capabilities after the printing process. Various stem cells have demonstrated the ability to preserve their differentiation potential and also have been directed by various cues applied during the printing process [143,144]. This section will highlight a few of the tissue-specific applications that have been studied with bioprinting technologies.

Bone

Bone regeneration is a natural target application for bioprinting given the importance of anatomical structure to its in vivo functions. Conventional 3D printing technologies are already in use clinically as patient-specific metal implants [145]. Bioprinting offers a unique and promising alternative to bone grafting given the wide variety in anatomic location, defect size, and patient-specific morphology for bone pathologies [146,147]. Bioprinting's advantage is especially apparent for bone defects that also feature a significant cosmetic function such as in craniofacial reconstruction [148]. Fedorovich et al. printed a mixture of Matrigel and alginate hydrogels with endothelial

TABLE 74.2 Three-dimensional bioprinting technologies for tissue regeneration applications.

Tissue/organ	Testing model	Printing method	Cell type	Bioink	Outcomes	References
Bone	In vitro viability and differentiation studies	Extrusion	Endothelial progenitor and multipotent stromal cells	Matrigel and alginate hydrogels	Viability and differentiation capability were unaffected by the printing process, and the two distinct cell populations were maintained within a single scaffold	[119]
	In vitro differentiation studies	Inkjet	Primary muscle-derived stem cells	Fibrin	Incorporation of BMP-2 caused spatially controlled osteogenic lineage differentiation even in myogenic media conditions	[120]
	In situ bioprinting	Laser	Osteoblasts	Glycerol and *n*-Ha slurry	Successful in situ bioprinting into mouse calvarial defects with minimal side effects	[121]
	In vitro	Extrusion	—	PCL/ hydroxyapatite	Reconstructed from CT scans, anatomically accurate and supportive of physiological loads	[122]
	In vitro degradation, mechanical, and cytotoxicity	Laser	Fibroblasts	PPF	Scaffolds maintained their mechanical stability, and degradation products did not induce significant cell death	[123]
	In vitro and in vivo bone formation	Extrusion	Mesenchymal stromal cells	PCL/PLGA/β-TCP	Scaffolds which were decellularized after brief culture period induced a greater bone formation in vivo	[124]
Cardiac muscle	In vitro	Extrusion	Spheroids of HUVECs and cardiac cells	Type 1 collagen	Viable cells, fusion and beating at 70 h with early signs of vascularization	[125]
	In vitro	Inkjet	Cardiomyocytes	Alginate	Viability in thickness as high as 1 cm and contraction was observed at macro- and microscopic level	[74]
	In vivo cardiac infarct patch	Laser	HUVECs and human MSCs	Poly(ester urethane urea)	Increased function and vessel formation compared to cell only treatment 8 weeks postinfarct	[16]
	In vitro	Extrusion	Cardiac-derived cardiomyocyte progenitor cells	Alginate	Cells demonstrated viability, phenotypic cardiac expression, and the ability to migrate from the hydrogel	[126]
Cardiac valve	In vitro	Extrusion	Porcine aortic valve interstitial cells	PEGDA and alginate	Anatomical accuracy was confirmed, a range in mechanical properties was obtainable by varying the concentrations of the hydrogels	[10,30,127]
Cartilage	in vitro	Laser	MSCs	None	Good structural integrity and osteoblast and chondrogenic differentiation	[128]
	in vitro	Inkjet	Articular chondrocytes	PEGDMA	Similar mechanical and biochemical properties of native cartilage and good integration with surrounding tissue. FGF-2 and TGF-β1 synergistically improved GAG deposition	[129,130]
	In vitro and in vivo	Inkjet	Rabbit elastic chondrocytes	Fibrin–collagen	Combined PCL electrospinning and bioprinting technique facilitated type 2 collagen and GAG deposition with improved mechanical properties	[44]
	In vitro	Extrusion	Chondrocytes	Alginate	Biomimetic ear could translate sound waves into an electrical signal and coexist with viable chondrocytes	[131]
	In vitro	Extrusion	Chondrocytes and adipocytes	PCL and PEG (sacrificial) and alginate (encapsulation)	Chondrogenesis and adipogenesis confirmed by immunostaining	[132]

Skin	In situ bioprinting	Extrusion	Amniotic fluid-derived stem cells	Fibrin–collagen	Amniotic fluid derived stem cells outperformed both MSCs and acellular graft	[133]
	in vitro and in vivo	Laser	Fibroblasts & keratinocytes	Collagen	Early indicators of stratum corneum formation and blood vessels after 11 days	[134,135]
	In vivo	Inkjet	Fibroblasts, keratinocytes, and microvascular endothelial cells	Collagen	10% improvement of wound contraction compared to allogeneic skin substitute	[136]
Bone–cartilage	In vitro and in vivo	Extrusion	MSCs and chondrocytes	Alginate	Distinct tissue regions were found after 21 days in culture and 6 weeks after subcutaneous implantation	[137]
	In vitro	Extrusion	Osteoblasts and chondrocytes	Type 1 collagen and HA	Cells showed better proliferation, migration, and function on hydrogels made from their native ECM and performed well in 14-day co-culture	[64]
Muscle–tendon	In vitro	Extrusion	Myoblasts amd 3T3 fibroblasts	PCL and PU	Cells were viable after a week in culture and scaffolds showed an appropriate trend in mechanical properties	[138]
Pancreas	In vivo	Extrusion	INS1E β/islets	Alginate and gelatin	Scaffolds were formed and embedded while maintaining cell viability & morphology	[139]
Adipose	In vitro	Laser	Adipose-derived stem cells	Alginate	Cells maintained viability, differentiation potential, and adipogenic gene expression after 10 days	[15]
Neural	In vivo	Extrusion	Bone marrow MSCs and Schwann cells	–	Grafts underperformed autograft controls, but provide a proof-of-concept for future work	[140]
	In vitro	Inkjet	Retinal ganglion cells and glia	–	Good cell viability and growth properties of cells was found after printing	[141]
	In vitro and in vivo	Laser	Neuronal, Schwann, and dorsal root ganglion cells	PEG	After three weeks, the nerve guide supported re-innervation across a 3 mm gap equal to that of an autograft	[142]

BMP-2, Bone morphogenic protein-2; β-TCP, beta-tricalcium phosphate; CT, computed tomography; ECM, extracellular matrix; FGF-2, fibroblast growth factor-2; GAG, glycosaminoglycan; HUVECs, human umbilical vein endothelial cells; HA, hyaluronic acid; MSCs, mesenchymal stromal cells; n-Ha, nano-hydroxyapatite; PCL, polycaprolactone; PLGA, poly(lactide-co-glycolide); PEGDA, poly(ethylene glycol) diacrylate; PEGDMA, poly(ethylene glycol) dimethacrylate; PEG, poly(ethylene glycol); PPF, poly(propylene fumarate); PU, polyurethane; TGF-β1, transforming growth factor beta-1.

progenitor cells and MSCs [119]. The constructs were implanted subcutaneously to immunodeficient mice. They were able to demonstrate that the incorporation of biphasic calcium phosphate microparticles caused the MSCs to differentiate into an osteogenic lineage and caused bone formation within 6 weeks after implantation. Phillippi et al. used the inkjet bioprinting to pattern bone morphogenic protein 2 on primary muscle-derived stem cells on fibrin-coated coverslips [120]. The stem cells differentiated into an osteogenic lineage even in myogenic differentiation media conditions. Keriquel et al. demonstrated in situ bioprinting by delivering nanoscale hydroxyapatite and osteoblasts into mouse calvarial defects with positive outcomes [121]. Using CT scanning, Yao et al. were able to print anatomically accurate, PCL/hydroxyapatite mandible scaffolds that supported physiological loads [122]. Wang et al. examined the degradation profile of printed PPF scaffolds as it pertained to pore size, porosity, and mechanical properties [123]. They also developed a novel test for cytotoxicity of the degradation products and determined the scaffolds to be suitable for bone tissue engineering applications. In order to incorporate biological materials with bioprinting technology, Pati et al. cultured MSCs on a printed PCL/PLGA/β-tricalcium phosphate (β-TCP) scaffold [124]. The cells deposited ECM during a brief culture period, after which the scaffold was decellularized. In vivo the ECM enriched scaffolds induced greater bone formation than unadorned scaffolds of the same composition.

Many limitations still exist including for large-sized defects and in high load-bearing applications. The lack of perfusion and neovascularization prevents large defects from being treated with bioprinting strategies, and further research in this area is needed [149]. In addition, the discovery of new bioprinting compatible materials and unique structural designs could increase the maximum load-bearing applications for these constructs. More work is also needed to closely match the degradation profiles of scaffolding materials with that of the bone remodeling rate. New bone formation is obstructed if the scaffolding material degrades too slowly, but the defect site is left without a load-bearing material if degradation occurs too quickly, damaging nearby tissue. Altogether, bone tissue is one of the more promising target tissue applications for bioprinting due to its many advantages relative to other tissue engineering strategies and the natural ability of bone to remodel in vivo.

Cartilage

Articular cartilage is imperative to reducing friction and absorbing compressive forces in load-bearing joints with little to no capacity for self-regeneration. Current cartilage tissue–engineering strategies are insufficient for reproducing tissue that is equivalent to healthy cartilage [150]. However, recently greater interest has been placed on the zonal differences found in cartilage matrix and cellular composition [151]. Bioprinting presents as an appealing tool for constructing stratified scaffolds, especially in patient-specific size and shape of individual lesions [152]. Gruene et al. used the LIFT to generate MSC grafts, showing good cell viability, density, and functionality [128]. MSCs were able to differentiate into osteoblasts and chondrocytes and the graft maintained good structural integrity in vitro. Cui et al. loaded chondrocytes into poly(ethylene glycol) dimethacrylate hydrogel and inkjet bioprinted them into an osteochondral plug [129]. The implant had mechanical and biochemical properties similar to native cartilage, and Safranin-O staining revealed good integration with surrounding cartilage tissue. The same group also used their experimental setup to investigate the effects of fibroblast growth factor 2 and transforming growth factor β1 on cartilage generation. Samples were cultured up to 4 weeks, and the highest GAG content was found for samples containing both growth factors, suggesting a synergistic effect between increased cell proliferation and increased chondrogenic phenotype expression [130]. To address some of the limitations of bioprinting, Xu et al. alternated between inkjet bioprinted layers of rabbit elastic chondrocytes suspended in a fibrin–collagen hydrogel and electrospun PCL [44]. The construct formed cartilage-like tissues in vitro and in vivo demonstrated by type II collagen and GAG deposition. In addition, the scaffolds with electrospun layers showed improved mechanical properties compared to scaffolds that were bioprinted only.

In particular, engineering the external ear has been a notably successful area of bioprinting cartilage tissue. The ear is almost completely avascular and aneural, has a complex geometry, and serves a largely aesthetic function which places a greater emphasis on individualizing each prosthetic to the specific patient. Mannoor et al. developed a bionic ear that can translate sound waves into an electrical output [131]. The scaffold was extrusion bioprinted with sodium alginate, silver nanoparticles, and chondrocytes in an ear-shaped geometry around the conductive, sound translating coil. Lee et al. extrusion printed PCL with PEG as a supporting sacrificial layer [132]. Chondrocytes and adipocytes were differentiated from adipose-derived stromal cells, encapsulated in alginate hydrogel, and dispensed into their respective regions. After 7 days in vitro culture, immunostaining analysis confirmed chondrogenesis and adipogenesis. Kang et al. also applied the extrusion-based bioprinting to fabricate a complex shape by making human-sized ear cartilage tissue construct (Fig. 74.5A) [1]. After implantation the printed ear shape was well maintained, with cartilage tissue formation upon gross examination. Histological

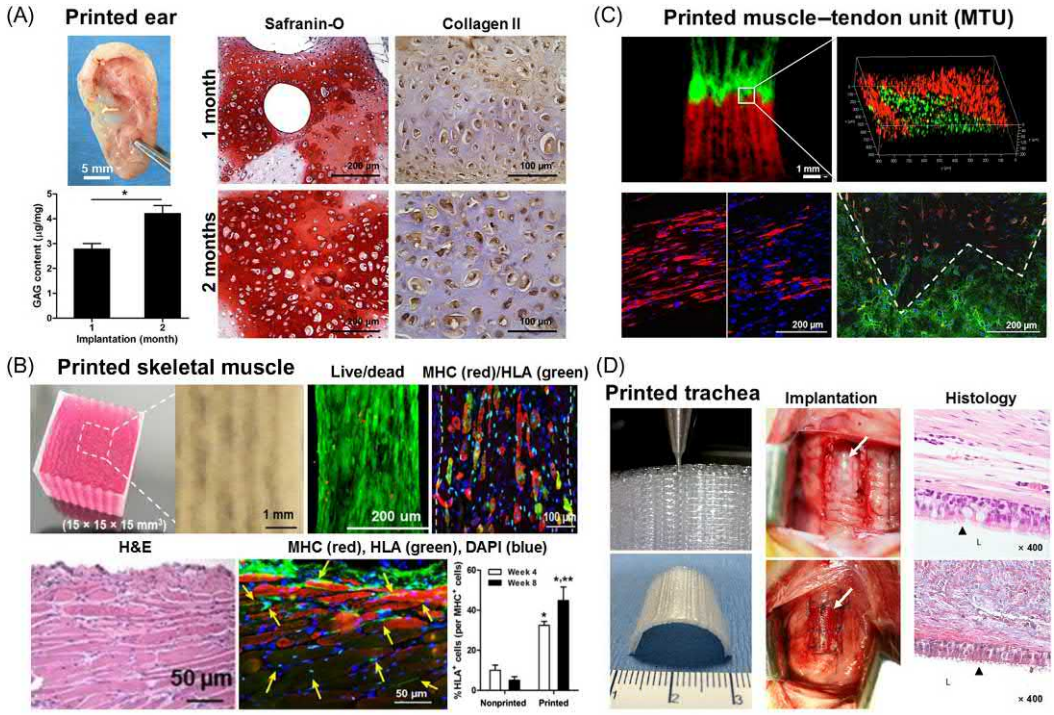

FIGURE 74.5 (A) Bioprinted ear construct: the shape was well maintained with substantial cartilage formation upon gross examination. Histological and immunohistochemical analyses showed the typical cartilage tissue formation [1]. (B) Bioprinted human skeletal muscle construct: the retrieved muscle constructs showed well-organized muscle fiber structures, vascularization, and host nerve integrity. The muscle force was recovered by up to 85% of native muscle at 8 weeks after implantation [153]. (C) Bioprinted MTU: fluorescently-labeled dual-cell printed MTU constructs (green: DiO-labeled C2C12 cells; red: DiI-labeled NIH/3T3 cells; yellow: interface region between green and red fluorescence) [138]. (D) Bioprinted tracheal construct: ingrowth of ciliary respiratory epithelium from the normal region was observed in the lumen of the bioprinted construct. Typical morphologies of respiratory mucosa and pseudocolumnar ciliary epithelium with goblet cells were well developed at 8 weeks after implantation (L: lumen) [154]. *MTU*, Muscle–tendon unit.

analyses showed the tissue formation of cartilage tissue as confirmed by GAG and collagen type II staining. Quantitatively, GAG content increased over time, reaching 20% of that of native ear GAG content at 2 months after implantation.

At this stage the next challenge for bioprinting as a means for cartilage regeneration is conducting translational studies. Very few in vivo studies have been conducted. Long-term stability of bioprinted cartilage constructs has yet to be demonstrated, and no studies have compared these strategies with practices currently used clinically. However, the research in cartilage bioprinting is growing exponentially and exhibiting many promising results for the future.

Skeletal muscle and tendon

The organized ultrastructure of skeletal muscle is required for muscle contraction and force generation [155]. Because 3D bioprinting mechanism enables to control of the spatial organization of cell-laden bioinks, Kim et al. were able to fabricate highly oriented muscle-like bundles for engineering skeletal muscle construct (Fig. 74.5B) [153]. A human muscle progenitor cell (hMPC)–laden hydrogel bioink was extruded with sacrificial acellular gelatin hydrogel bioink and a supporting PCL pillar. The printed hMPCs were longitudinally aligned along the printed pattern direction and showed high cell viability (over 90%) at 5 days in vitro. In the in vivo study where the printed muscle structures were subcutaneously implanted in athymic mice, the von Willebrand factor vessels and neurofilament nerves were observed at 2 weeks after implant. Furthermore, this bioprinted skeletal muscle construct maintained the tissue organization, followed by the tissue maturation and host nerve integration in rat tibialis anterior muscle defect model [153]. The results demonstrated that the 3D bioprinting was capable to produce promising structural and functional characteristics of skeletal muscle constructs in vitro and in vivo.

Tendon has a hierarchical architecture, and tenocytes are aligned along with dense collagen fibrous structure [156]. To mimic these structural characteristics of the tendon, an electrohydrodynamic jetting printing was introduced to generate a tubular-shape, multilayered tendon construct, having high porous, oriented microscale PCL fibers [157]. The cultured human tenocytes on the

bioprinted structure showed high cellular orientation, metabolism, and type I collagen expression. Importantly, 3D bioprinting technologies are particularly useful for composite tissue constructs like muscle–tendon. Merceron et al. used an integrated tissue and organ printing system to print four different components for the fabrication of a single integrated muscle–tendon unit (MTU) construct (Fig. 74.5C) [138]. The printed MTU construct comprised mechanically heterogeneous polymeric materials that were elastic (polyurethane, PU) on the muscle side and relatively stiff (PCL) on the tendon side, in addition to having a tissue-specific distribution of cells with C2C12 myoblasts on the muscle side and NIH/3T3 fibroblasts on the tendon side. The results showed that cells were printed with high cell viability and cellular orientation as well as increased musculotendinous junctional gene expression. It is demonstrated that 3D bioprinting technology enables a 3D heterogeneous tissue construction having region-specific biological and biomechanical characteristics.

Cardiac tissue and heart valves

The heart is a complex organ in both shape and tissue organization, both of which are difficult to replicate by other fabrication methods. The ability to spatially control the distribution of different cell types and growth factors makes bioprinting an attractive option for cardiac engineering, although only proof-of-concept successes have been accomplished thus far [158]. Jakab et al. were able to use extrusion-based bioprinting to pattern spheroids of HUVECs and cardiac cells on collagen [125]. The cells proved viable after the process, fused at 70 hours into a beating tissue, and showed early signs of forming vascularization. Using inkjet bioprinting, Xu et al. printed a half heart shape (with two connected ventricles) with cardiomyocytes encapsulated in an alginate hydrogel [74]. Cell viability was preserved in constructs as thick as 1 cm due to designed porosity within the structure, and contraction was observed in vitro at both microscopic and macroscopic levels. Instead of targeting whole heart reconstruction, Gaebel et al. developed a cardiac patch for regeneration after cardiac infarction [16]. Using LIFT bioprinting, HUVECs and human MSCs were patterned on polyester urethane urea and transplanted into the infarct zone. After 8 weeks, increased vessel formation and function were found compared to control treatment of bioprinted cells alone. A patch by Gaetani et al. used human cardiac-derived cardiomyocyte progenitor cells and extrusion bioprinted them in a sodium alginate mesh pattern [126]. They demonstrated cell viability, phenotypic expression of cardiac lineage, and the ability to migrate from the alginate, suggesting that bioprinting can be used for defined cardiac cell delivery. Wang et al. demonstrated the feasibility of 3D bioprinting to bioengineer a functional cardiac tissue construct that possesses a highly organized structure with unique physiological and biomechanical properties similar to native tissue [159].

Patients with heart valve failure must receive a replacement valve which can either be mechanical, require a lifetime of anticoagulant treatment, or biological, which typically fail within 10–20 years [160]. Bioprinting has gained momentum as a potential heart valve fabrication strategy to mimic the complex geometry and nonhomogenous material makeup, mechanical properties, and cell distributions which naturally occur in heart valves [160]. Hockaday et al. used a dual ionic and physical cross-linking hydrogel strategy by using PEGDA sodium alginate composite [10]. Printing accuracy of aortic valve root wall and tri-leaflets was confirmed via micro-CT scanning. By varying PEGDA and alginate concentrations, elastic moduli were found to range from 1.5 to 5.3 kPa. Porcine aortic valve interstitial cells were seeded and cultured on the scaffold for 21 days with nearly 100% viability. Later studies by the same group printed the cells directly within the hydrogel as opposed to seeding the scaffolds afterward, also with good geometric accuracy, cell viability, and mechanical properties [30,127]. While these studies are far removed from use in the clinic, they demonstrate that bioprinting technology is amenable to cardiac tissue regeneration and open the door for many future studies focused on improving the current methodology and outcomes.

Skin

Bioprinting is an excellent technology for the deposition of distinct layers and has been used in an attempt to mirror the layers of native skin, and research in this area has increased significantly recently. Skardal et al. performed directly in situ printing of amniotic fluid-derived stem cells suspended in the fibrin–collagen hydrogel [133]. In comparison to an acellular graft and an MSC graft, amniotic fluid-derived stem cells showed increased microvessel density and capillary diameter. Laser-assisted bioprinting has been used to embed fibroblasts and keratinocytes in collagen [134]. Histology revealed a high density of both cell types and the expression of laminin protein. The same group grafted their construct onto mice and reported early indicators of stratum corneum formation and blood vessels after 11 days [135]. Yanez et al. printed keratinocytes and fibroblasts in collagen as well but also included human microvascular ECs [136]. When implanted onto the backs of mice and compared to allogeneic skin substitute as a control, wound contraction improved by 10% and histological results appeared similar to that of normal skin. Sweat glands and hair follicles remain elusive, as does commercial and regulatory viability [161]. Nonetheless,

skin bioprinting has shown many encouraging successes, and the clinical bioprinting of skin appears to be an impending reality [42]. Very recently, Albanna et al. validated a mobile skin bioprinting system that provides rapid on-site management of extensive wounds. This system could facilitate the precise delivery of epidermal keratinocytes and dermal fibroblasts directly into a skin wound area, replicating the layered skin structure [162].

Other tissue types

Many other tissue types have been targeted with bioprinting technology, albeit to a lesser extent than those discussed thus far. This could be caused by a lesser clinical need, a higher difficulty of tissue engineering in general, or a poor matchup between the advantages of bioprinting and the necessary components for regenerating that tissue. Composite tissues are a major challenge facing regenerative medicine. No organ in the body is completely isolated, and many tissues such as tendons have specific and functional interfaces with other tissue types. Bioprinting is uniquely positioned to address this problem by spatially directing the placement of different cell types, growth factors, and biomaterials [163–165]. Fedorovich et al. extrusion bioprinted MSCs with hydroxyapatite, β-TCP, and biphasic calcium phosphate particles in alginate for one section of the scaffold and chondrocytes in alginate for the other [137]. Distinct tissue formation was found after 21 days in a mixture of osteogenic and chondrogenic media culture as well as after 6 weeks of subcutaneous incubation in vivo. Park et al. bioprinted osteoblasts in collagen I hydrogel and chondrocytes in HA hydrogel with good results after 14 days in vitro, in the process showing that the cells performed better on hydrogels made from their native ECM [64]. Finally, Merceron et al. targeted the MTU using PCL and 3T3 fibroblasts for the tendon zone and PU and myoblasts for the muscle zone [138]. The results showed that the cells were printed with good viability, begin developing into highly aligned morphology characteristic of muscle and tendon, and had increased muscle-tendon junction (MTJ)-associated gene expression during the culture.

Neural tissue has additionally been addressed by bioprinting. Owens et al. developed a nerve graft containing bone marrow MSCs and Schwann cells using the extrusion bioprinting [140]. The grafts were implanted for 10 months in a rat sciatic nerve injury model with autograft controls, concluding that that bioprinting was a promising approach to nerve grafting. Retinal ganglion cells and glia were piezoelectric inkjet bioprinted by Lorber et al., showing good cell viability and growth promoting properties in vitro [141]. Pateman et al. used a microsterolithographic technique to print a PEG-based nerve guidance channel for nerve repair [142]. At 3 weeks the nerve guidance was capable of supporting reinnervation across a 3-mm injury with similar results to that of an autograft in a common fibular nerve injury mouse model.

The trachea mainly comprises tightly stacked cartilage rings and respiratory mucosa in the luminal surface. Several synthetic implants have been used to reconstruct tissue defects [166–168]; however, these implants have been limited in their ability to mimic the tracheal functions biologically and biomechanically. Jung et al. developed a biomimetic tracheal construct using 3D bioprinting approach that could reconstruct a partial tracheal defect in a rabbit model (Fig. 74.5D) [154]. The printed tracheal PU constructs provided excellent structural characteristics. In the rabbit tracheal defect model, the printed PU constructs maintained the biomechanical function of the trachea, while the microscale porous architecture in the construct allowed the cellular infiltration for the biological integration with host tracheal tissue. Moreover, the printed PU scaffold provided a proper microenvironment to facilitate the resurfacing of the ciliated respiratory epithelium and the ingrowth of connective tissue with microvasculature.

Gruene et al. [15] laser bioprinted ASCs encapsulated in alginate-based bioink for adipose tissue engineering. They proved that the cells maintained their viability, differentiation ability, and adipogenic gene expression after 10 days in culture. Preliminary progress has also been made in several more complicated organs such as the intestine [169,170] and pancreas [139]. This section briefly examined the application of bioprinting to specific tissue types. Many studies were excluded due to space constraints and several tissue types, which have been explored via bioprinting, have not been covered here.

Conclusion and future perspectives

The principle of tissue engineering aims to build patient-tailored tissue constructs to reduce patient morbidity and mortality while improving quality of life. Currently, 3D bioprinting technologies combined with this principle hold great promise to achieve this goal. The focuses on replicating complex and heterogeneous tissue constructs continue to increase as 3D bioprinting technologies progress. Progression from single simple tissues, such as skin, bone, and cartilage, to organized contractile tissues, such as skeletal muscle and cardiac tissue, to composite tissues, such as osteochondral tissue and MTU, and finally to robust solid organs such as the kidney and heart are underway [2].

The clinically relevant workflow of 3D bioprinting requires multiple components that mainly include medical imaging, 3D CAD/CAM, 3D printing process, bioink materials, and translational cell source. Among those, various novel bioink systems have been developed for improving printability with high-resolution capability and

structural integrity. Advanced biomaterials, including hydrogels and polymers, that can serve as cell delivery bioinks and supporting structures but which also provide biological properties and mechanical and structural support, are required for 3D bioprinting. Moreover, advances in biomaterials depending on 3D bioprinting mechanisms are necessary for the long-term success in tissue engineering applications. Recently, an approach that utilizes the decellularized ECM can provide tissue-specific microenvironment to the cells. This decellularized ECM-based bioinks are still the closest biological microenvironment that mimics in vivo conditions; thus tissue-specific ECM-based bioinks are capable of providing critical cues for targeted cell engraftment, survival, and tissue formation.

There must be an increase in knowledge of biological, anatomical, and physiological aspects of complex tissues and organs. In particular, the development of in vitro 3D tissue models to study tissue- or organ-specific functions in the body will require a better understanding of morphological, structural, and functional units in tissues or organs. For clinical tissue engineering, the well-known limitation to build a large-scale tissue construct is vascularization in the construct. 3D bioprinting strategies have continued to overcome this limitation in various tissue engineering applications. A few groups have made progress toward printing vascularized tissue constructs; however, integrating functional microvascular structures into tissue- or organ-like constructs has not been accomplished. Approaches to utilize high porosity, angiogenic factors, and highly organized patterns of vascular cells by 3D bioprinting technologies may improve the principle of vascularization in the tissue-engineered constructs.

3D bioprinting technologies offer the opportunity to reconstruct the structural and ultimately the functional complexity of human tissues that incorporate multiple cell types, biomaterials, and bioactive molecules, resulting in sophisticated tissue constructs that have the potential to replace damaged or diseased human tissues and organs. Though there is much work to be accomplished to advance these technologies toward successful clinical translation, our efforts will constantly contribute to produce clinically applicable tissue constructs until 3D bioprinting strategy is able to improve the lives of patients. We envision that the bioprinted tissue constructs such as skin, bone, and cartilage have the great potential to be translated to clinical applications within a short period of time.

Abbreviations

2D	two-dimensional
3D	three-dimensional
ASCs	adipose-derived stem cells
β-TCP	beta-tricalcium phosphate
CAD	computer-aided design
CAM	computer-aided manufacturing
CT	computed tomography
DNA	deoxyribonucleic acid
ECM	extracellular matrix
FDA	US Food and Drug Administration
GAG	glycosaminoglycan
GelMA	gelatin methacrylate
HA	hyaluronic acid
HUVECs	human umbilical vein endothelial cells
HUVSMCs	human umbilical vein smooth muscle cells
LIFT	laser-induced forward transfer
MSCs	mesenchymal stem cells
PCL	poly(ε-caprolactone)
PEGDA	poly(ethylene glycol) diacrylate
PEGDMA	poly(ethylene glycol) dimethacrylate
PLGA	poly(lactide-*co*-glycolide)
PPF	poly(propylene fumarate)
UV	ultraviolet

Glossary

Bioprinting	The incorporation of biological materials into additive manufacturing techniques, either by directly depositing cells layer-by-layer or indirectly by 3D printing biologically active materials for later use in cellular applications.
Cross-linking	A chemical bond between two polymer chains which changes the overall properties of the material.
Electrospinning	A method for the production of fibers which utilized electrical forces to draw out nanoscale threads of melted polymer material.
Extracellular matrix	The environment secreted by cells which biochemically and structurally supports a cellular network.
Extrusion bioprinting	Direct contact bioprinting mechanism that relies on pressure or displacement to force material through the syringes.
Fused deposition modeling	An additive manufacturing technology which extrudes heated material layer by layer to create 3D structures.
High-throughput screening	Drug delivery process in which a lot of drugs or chemicals can be tested at a rapid pace.
Hybrid bioprinting	Utilizing multiple bioprinting mechanisms in one system to overcome the limitations of each mechanism.
Hydrogel	A polymeric, gel material in which the main component is water.
In situ bioprinting	Bioprinting directly in vivo such as onto a skin wound or into a bone defect; As opposed to bioprinting separately and then surgically placing a scaffold into a defect.
Jetting bioprinting	Originating from inkjet printers, this noncontact bioprinting mechanism uses pressure pulses to apply bioink in predetermined locations.

Laser-assisted bioprinting	Bioprinting mechanism that uses a focused laser to generate high-pressure bubbles that propel cell-containing material onto a substrate.
Microfluidic device	A device which is able to manipulate and control the flow of fluids on a microliter to picoliter scale.
Scaffold	The material which acts in place of the ECM, providing a physical, 3D environment for cells to attach, migrate, and proliferate.
Micropatterning	Precisely controlling the cellular microenvironment on a substrate for the purposes of studying cell behavior.
Perfusion	The process of oxygen and other vital nutrients being delivered from the bloodstream to tissues and cells.
Piezoelectric	A ceramic crystal which creates an electric charge in response to an applied mechanical stress.
Printability	The ability and usefulness of a particular material to be applied as a bioink.
Printing resolution	The smallest dimension which can be controlled by a particular bioprinting system.
Spheroid	A 3D conglomerate of cells, often organized into a sphere-shape.
Stereolithography	3D printing process in which liquid photopolymer is exposed above a perforated platform and then cross-linked by a UV laser forming the first layer. The platform then lowers exposing a new surface of liquid which the UV laser crosslinks to form layer two.
Structural stability	The ability of a printed construct to maintain its shape.

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Chapter 75

Biofabricated three-dimensional tissue models

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Introduction

Over the past decade, three-dimensional (3D) biofabrication has combined principles of 3D bioprinting and 3D bioassembly to replicate complex tissue structure and function [1]. Using new advancements in 3D printing technology, biomedical engineers can control the spatial positioning of biomaterials (natural, synthetic), biochemicals (drugs, growth factors), and/or living cells [induced pluripotent stem cells (iPSCs), mature-differentiated cells, embryonic stem cells, etc.]. As such, this approach enables the creation of precise tissue-engineered (TE) models for drug screening, studying human development, fabrication of functional implants to replace damaged tissue, and delivery of biomolecules with temporal and spatial cues to guide autologous tissue regeneration in vivo. These transformative tools have allowed researchers to develop 3D models that accurately depict the structure and function of normal and diseased tissues, resulting in more physiologically relevant behavior from the cell to the whole tissue level than traditional two-dimensional (2D) model systems.

2D cell culturing has been used for over a century to study cellular responses to biophysical and biochemical stimulation [2]. However, many of these cell responses differ in 2D culture than in vivo, making them poor-to-moderate platforms for various tissue models. To overcome this, 3D model platforms have been utilized that more accurately mimic the natural complex biophysical and biochemical stimuli cells experience in vivo. Studies have routinely demonstrated that cells in 3D culture differ morphologically and physically from cells cultured in 2D, which affects function, signaling, and gene expression [3–6]. Early evidence of this phenomenon was reported in 1989, when Dunn et al. demonstrated that hepatocytes cultured "sandwiched" between two layers of collagen gel maintained normal morphology and albumin secretion for at least 42 days, while hepatocytes cultured on a single collagen layer ceased albumin secretion within a week [7]. This early work and similar studies have motivated developing biomimetic 3D environments in order to preserve normal cellular function.

3D tissue models can be separated into two categories: (1) scaffold-based and (2) scaffold-free designs. Scaffolds serve as a biomimetic extracellular environment to provide chemical stimulation and/or mechanical support, in order to promote natural cellular interactions and organization. Scaffolds often consist of specially designed materials and are designed to degrade over time, coinciding with de novo extracellular matrix (ECM) production. These scaffolds can consist of one of more materials, in order to simultaneously provide a mechanically stable structure for handling and an environment which promotes cell adherence and migration. Cells can either be directly incorporated into the scaffold, or scaffolds can rely on the autologous cellular environment to migrate and differentiate within when implanted in vivo. Challenges with scaffold-based models include inhomogeneous distributions of cells within the scaffold and lack of gross complex tissue self-assembly. Scaffold-free models rely on self-assembly of cells into larger constructs with autologous ECM deposition for support. The building blocks of these constructs are often cell sheets, spheroids, and tissue strands and do not require complex fabrication

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techniques, which are often utilized for scaffold-based models. For example, tissue strands can be formed by injecting a cell pellet into a microtubular capsule, allowing those cells to spontaneously adhere to one another and remodel via cadherin-mediated cell−cell interactions [8,9]. These strands can then be 3D printed to form TE constructs such as tissue patches. Scaffold-free models are limited by size (they often form a necrotic core due to inadequate nutrient diffusion) and have inadequate mechanical properties, leading to cell damage when handled [10]. Recently, synergistic methods combining scaffold-based and scaffold-free tissue models have been proposed, which may allow for more complex, mechanically stable TE constructs. Silva et al. reported the use of lockyballs—mechanically interlockable microscaffolds—in order to provide a sound mechanical structure, within which spontaneous tissue organization was allowed to occur [11]. Prefabricated human adipose stem cell spheroids were seeded into lockyballs and allowed to self-assemble and differentiate toward adipogenic, osteogenic, and chondrogenic lineages, effectively combining scaffold-based and scaffold-free approaches.

Native tissues are made up of a heterogeneous cell population consisting of fibroblasts, endothelial cells, and stem cell populations in addition to tissue-specific cells that cofunction to regulate ECM production, biochemical signaling, and overall tissue functionality. Traditional seeding of single or multiple types of cells on a scaffold is a simple approach; however, it is difficult to replicate the complex multicellular tissue composition that exists in vivo. Coculture platforms are of increasing interest as they provide both cell−cell (direct) and cytokine (indirect) interactions to occur, which result in more advanced stages of tissue formation [12]. Recent advances of 3D bioprinting now allow for precise spatial control over cellular deposition for both scaffold-based and scaffold-free tissue models. For example, Liu et al. demonstrated that hepatocytes alone will cease albumin and urea secretion after 2 weeks; however, secretion is protected when patterned with fibroblasts and endothelial cells in the same model even if not all cell types are in direct contact with one another [13]. Of particular interest is the patterning of endothelial cells within a construct to enhance vascularization [14] because as the engineered tissues grow, a necrotic core is likely to develop due to the diffusion limits of oxygen and nutrients to cells. Thus vascularized scaffolds would allow for larger and more clinically relevant engineered tissues to be fabricated.

3D biofabrication incorporates several powerful approaches to constructing complex tissue models. However, the appropriate choice of 3D bioprinting technology, biomaterials, and cells must be considered a priori in order to adequately represent the key mechanical, biochemical, and structural properties of the tissue of interest. The effective use of these tools has resulted in advanced 3D models of many tissue systems. In the following sections, we will discuss key factors involved in model design, and specific examples of how these techniques are applied to real-world situations.

Current methods of three-dimensional biofabrication

The most commonly used methods for 3D bioprinting include inkjet-based, extrusion-based, and light-assisted printing. All of these techniques have been used to either 3D print scaffolds onto which cells can be seeded or to directly encapsulate cells to form complex tissue models. The preprinted material is termed "bioink" and can consist of cells, biochemicals, and/or biomaterials, depending on the intended structure and printing method. Each of these methods has strengths and weaknesses that must be considered. Below, we will provide a detailed description, evaluation, and comparison of each of these techniques. In addition, these techniques are directly compared in Table 75.1.

Inkjet printing

The earliest forms of bioprinting systems were modified conventional desktop inkjet printers. Inkjet printers are able to deposit a very small volume (1−300 pL) of liquid onto a substrate through small nozzles. Inkjet bioprinters normally utilize a bioink consisting of cells in either culture medium or a cross-linkable hydrogel or can be acellular. Current inkjet-based bioprinters can print at speeds in the range of hundreds of millimeters per second and deposit hundreds of thousands of droplets per second, with a resolution as high as 50 μm [15]. The limit of resolution of inkjet printers relies both on the diameter of the nozzle and the viscosity of the bioink, thus reducing the diameter of the nozzle alone increases the likelihood of clogging. This limits the types of materials that are able to be printed using this method to low-viscosity (∼30 mPa s) or water-based materials [54]. There currently exist three different approaches to inkjet printing (Fig. 75.1):

1. *Thermal*—Thermal inject printing heats a small volume of the bioink (up to 300°C) for a matter of microseconds to vaporize the liquid and inflate an air bubble to force the ink out of the nozzle head. This method is most commonly used as it has the highest cell viability after printing, is user-friendly, and generally inexpensive [19].
2. *Piezoelectric*—Piezoelectric inkjet printing uses a mechanical pulse generated by a piezoelectric actuator. While no difference in viability of piezoelectric printed versus unprinted fibroblasts has been found,

TABLE 75.1 Comparison of different three-dimensional bioprinting techniques.

	Inkjet printing	Extrusion printing	DLP printing	TPP printing	References
Printing process	Serial (drop-by-drop)	Serial (line-by-line)	Parallel and continuous (projection-based)	Serial (dot-by-dot)	[19–25]
Printing speed	Medium (mm/s)	Slow (10–50 μm/s)	Fast (mm^3/s)	Medium (mm/s)	[26–29]
Resolution	<1 pL droplets; 50 μm wide	>5 μm	1 μm	~200 nm	[15,29–32]
Biomaterials	Low-viscosity suspensions of cells, biomolecules, growth factors	Polymers (natural/synthetic), plastics, cells, proteins	Polymers (natural/synthetic), cells, nanoparticles	Polymers (natural/synthetic), cells, nanoparticles	[15,31–35]
Biomaterial requirements	Low viscosity, rheopectic behavior, nonfibrous, rapid cross-linking	Shear thinning, low surface tension, low adhesion, rapid cross-linking, shape retention	Photopolymerizable, contain low-toxicity photo-initiators, stability, high mechanical strength	Photopolymerizable, contain low-toxicity photo-initiators, stability, high mechanical strength	[36]
Biomaterial viscosity	3.5–12 mPa s	30 to 6×10^7 mPa s	1–300 mPa s	1–300 mPa s	[15,34,37–39]
Mechanical integrity	Poor due to interfaces	Poor due to interfaces	Excellent due to scanningless and continuous printing	Poor due to interfaces	[17]
Cell viability	>85%	40%–80%	85%–95%	>85%	[40–43]
Compatible biomaterials	Thermo/pH/photosensitive	Thermo/photosensitive	Photosensitive	Photosensitive	[44–47]
Printer construction	Simple	Moderate	Complex	Complex	[21,23]
Advantages	Wide availability, low cost, ability to introduce concentration gradients	Many printable materials, scaffold-free fabrication, easy to print multiple materials simultaneously	Fast, nozzle free, no contact, precise control of geometry, precise control of mechanical properties, high cell viability	Nozzle free, highest fabrication resolution, single cell manipulation	[1,15,21,33,48–51]
Disadvantages	Limited vertical structure, susceptible to clogging, thermal/mechanical stress on cells	Low viability, critical timing of polymerization, requires matching material densities to preserve shape	Moderate capital cost, material waste, limited number of biomaterials	High capital cost, limited to small structures, material waste, limited number of biomaterials	[1,22,27,33,52,53]

DLP, Digital light processing; *TPP*, two-photon polymerization.
Source: Adapted from Murphy SV, Atala A. 3D bioprinting of tissues and organs. Nat Biotechnol 2014;32:773–85 [15]; Li J, Chen M, Fan X, Zhou H. Recent advances in bioprinting techniques: approaches, applications and future prospects. J Transl Med 2016;14:271 [16]; Zhu W, Ma X, Gou M, Mei D, Zhang K, Chen S. 3D printing of functional biomaterials for tissue engineering. Curr Opin Biotechnol 2016;40:103–12 [17]; Jana S, Lerman A. Bioprinting a cardiac valve. Biotechnol Adv 2015;33:1503–21 [18].

there is a concern that the range of frequencies employed by these printers (15–25 kHz) can cause damage to the cell membrane and induce cell lysis [19,55].

3. *Electromagnetic*—Electromagnetic inkjet printing utilizes miniature solenoid valves to dispense fluid [56]. This method produces much larger drop volumes than other inkjet printing methods [57].

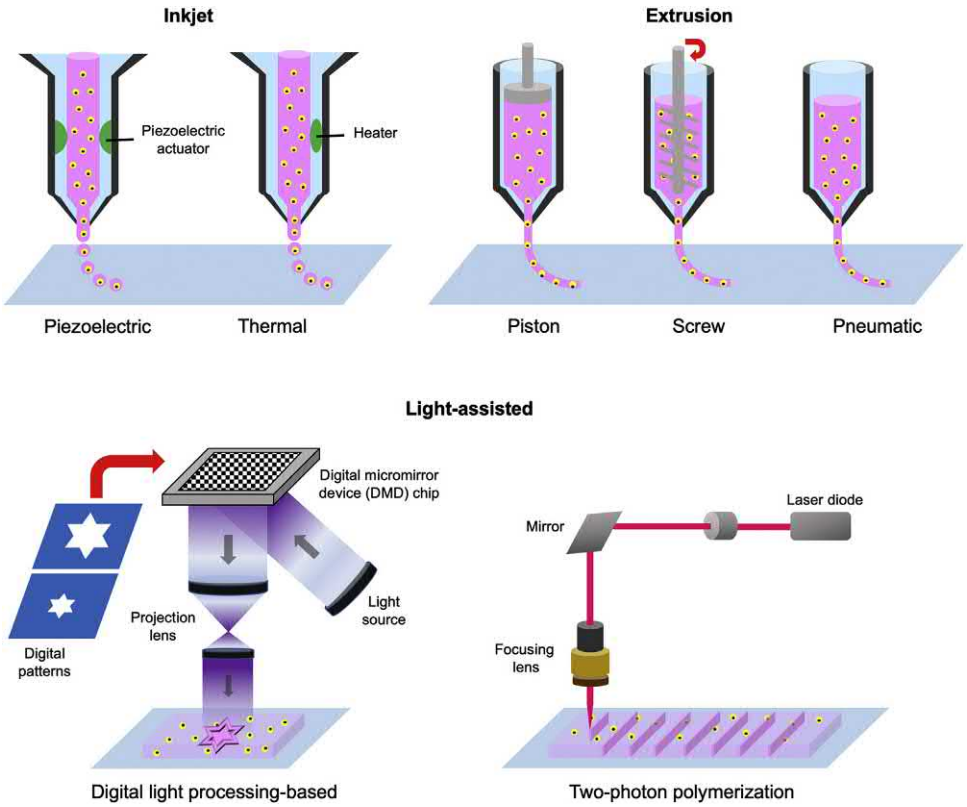

FIGURE 75.1 Schematic of different 3D bioprinting platforms.

While initially there was a lot of interest in inkjet bioprinting, there has been little development toward the fabrication of large tissue constructs. It is very difficult to layer low-viscosity liquid droplets on top of a solid surface with high fidelity. In addition, the deposition of cells results in high thermal or shear stress which can affect viability. While this method is cheap and flexible, challenges pertaining to bioink composition, resolution, layering, and printing speed must be resolved in order to build complex 3D models that other 3D bioprinting modalities are capable of.

Extrusion printing

Extrusion-based printers dispense bioink in a continuous manner at precise points in space. These bioprinters typically use piston-, screw-, or pneumatic-based controls to regulate the rate of bioink deposition, with linear actuators controlling the precise $x-y-z$ location of the nozzle or substrate (Fig. 75.1). The shape of the fabricated construct can be designed using traditional computer-aided design modeling software, which allows for designs inspired directly from computed tomography (CT) or magnetic resonance imaging (MRI) images.

A vast array of bioinks are available for use with this method including encapsulated cells, naturally derived polymers, synthetic hydrogels of varying viscosity, and sacrificial bioinks that can be washed away to leave voids. The viscosity of the bioinks available for extrusion printing is greater than that of inkjet printing, and allows for the fabrication of larger, mechanically stable structures. Certain bioinks can be thermally, chemically or photocrosslinked to form solid structures and increase handleability.

Several approaches have been taken in order to make models robust and complex, in order to more accurately replicate native tissue structure. These printers can be constructed with several nozzles, each containing a different bioink, which allows for multimaterial, multicellular constructs. This allows for both scaffold-based and scaffold-free tissue models to be fabricated using the same machine. In addition, coaxial printer heads allow for simultaneous bioprinting of a core bioink within a sheath bioink, which can be used to create microfibrous or hollow constructs.

There are a few limitations to extrusion-based 3D printing. Encapsulated cells undergo shear stress during extrusion, which limits cell viability. While extrusion-based 3D-printing allows for fabrication using more viscous mediums, these mediums often require more pressure, which affects cell viability more than the nozzle diameter, sometimes leading to worse cell viability than inkjet-based approaches [26]. While decreasing pressure

would increase cell viability, extrusion-based 3D bioprinting already has a very slow printing speed (10−50 μm/s). Typically, larger nozzle sizes are used which decreases resolution, but allows for overall larger structures to be fabricated. Therefore bioink viscosity, nozzle diameter, extrusion pressure, and printing speed must all be accounted for when fabricating cell-laden structures using this method. However, structures that do not require microscale features such as bone, cartilage, and skin can be easily fabricated using this method.

Light-assisted bioprinting

Light-assisted bioprinting is based on spatially controlling solidification of a liquid photopolymerizable material using light. This technique is of increasing interest in TE, as it allows for the encapsulation of cells with high viability, in addition to its rapid fabrication speeds, and high resolution, which altogether affords tight control over mechanical, physical, and chemical properties of the fabricated models. There are two main forms of light-assisted bioprinting: digital light processing (DLP)− and two-photon polymerization (TPP)−based bioprinting.

Digital light processing−based bioprinting

The basic components of a DLP-based bioprinting platform are a light source, a digital micromirror device (DMD) chip, a motorized computer-controlled stage, and a probe (Fig. 75.1). The light source (UV or visible) is reflected at the DMD chip, which contains 1−4 million micromirrors that can be rotated to be "on" or "off" using a simple binary mask. The light that is reflected off of the DMD chip passes through a series of optics into a solution of photopolymerizable biomaterials resulting in simultaneous printing of an entire plane of an optical pattern. By moving the stage or the light focal plane along the *z*-direction, complex patterns can be fabricated in 3D.

DLP-based bioprinters have several advantages over inkjet- and extrusion-based bioprinters, thus highlighting their versatility. Structures fabricated with DLP-based bioprinting are smooth and have higher mechanical integrity due to the lack of artificial interfaces between deposited materials that inherently exist in inkjet- and extrusion-based bioprinted constructs. Moreover, this method results in a significant time advantage and allows structures to be fabricated in a matter of seconds to minutes. The resolution of DLP-based printers is dependent upon the focal size of the reflected light beam and is usually on the order of a few microns. The stiffness of these structures can be modified by increasing power from the light source or by modifying the duration of exposure which affects the degree of polymerization of the fabricated structure. This can be used to create a single structure with multiple different stiffness profiles, which could more accurately depict models associated with pathology or disease. When printing cells, no shear forces are applied, resulting in higher cell viability than inkjet- or extrusion-based bioprinters. Taken together, DLP-based bioprinters can be used to fabricate complex 3D structures with fine features in a matter of seconds.

Two-photon polymerization−based bioprinting

TPP-based bioprinting utilizes a focused, near-infrared, femtosecond laser to polymerize a monomer solution (Fig. 75.1). Polymerization only occurs at the peak intensity area of the laser focal spot, where the energy is intensive enough to trigger nonlinear absorption of the femtosecond laser in the monomer solution and lead to photopolymerization for printing. This results in resolution below the diffraction limit and allows for high fidelity nanostructures with features less than 100 nm. 3D structures, including overhanging structures, can be fabricated by rastering through the monomer solution. The tradeoff of such a high-resolution printer is a decrease in the fabrication size and speed, although good cell viability is preserved. Similar to inkjet- and extrusion-based printers, since this is a noncontinuous printing process, fabricated structures have interfaces between photopolymerized parts, resulting in compromised mechanical integrity.

Both DLP- and TPP-based bioprinters suffer from few limitations. However, since no nozzles are used, the photopolymers typically reside in a reservoir from which the objects are printed and can result in wasted materials and increased cost. In addition, light-assisted printers utilize photopolymers, which must be chemically modified in order to facilitate 3D printing, thus limiting the number and type of materials which can be used.

Biomaterials for three-dimensional fabrication

Biomaterials are engineered substances that can be fabricated to interact with living biological systems either as therapeutics or diagnostics [58]. In order to fabricate physiologically relevant tissue models, it is necessary to closely mimic the natural physical and chemical properties of the tissue of interest. Therefore biomaterial selection is a crucial part of the design process and can govern which 3D fabrication methods may be used.

The biomaterials used in 3D bioprinting are often referred to as bioinks. These bioinks are most often hydrogels—a hydrophilic network consisting of cross-linked polymeric chains. Hydrogels can contain an aqueous solution of up to 1000 times its original weight [59]. The ability of hydrogels to absorb water arises from functional hydrophilic groups attached to their polymeric backbone and their ability to retain a structure is due to

the cross-links between their polymer chains [59]. Increasing cross-linking density increases the strength of a hydrogel, as well as decreasing the amount of water that can be absorbed. Due to its structure, the hydrogel typically retains high permeability to oxygen and nutrients, which makes it an attractive medium for 3D tissue scaffolds. 3D-printable biomaterials can be divided into two categories: (1) naturally derived and (2) synthetically derived. Many differences exist between the printability, biocompatibility, mechanical strength, and biochemical components of naturally and synthetically derived biomaterials. For a more detailed review of biomaterials used in 3D bioprinting, the reader is directed to the comprehensive reviews by Hospodiuk et al., Skardal and Atala, Parak et al., and Choudhury et al. [36,60–62].

Naturally derived biomaterials

Naturally derived bioinks that have been used for 3D tissue fabrication include collagen, gelatin, agarose, hyaluronic acid, silk proteins, chitosan, alginate, and decellularized ECM (dECM). Naturally derived biomaterials are an attractive bioink for 3D printing, as they contain the intrinsic biophysical and biochemical components of the native ECM. This results in increased cell adhesion, proliferation, differentiation, and migration. Structures made with naturally derived bioinks generally have high biocompatibility when integrated with native host tissues. The main detraction to naturally derived biomaterials is that they are often mechanically weak on their own and difficult to print with precision. Therefore naturally derived biomaterials are sometimes combined with synthetic biomaterials to form composites. In addition, as naturally derived biomaterials are derived from actual tissues, they are susceptible to batch-to-batch variability, which effects model consistency. Nearly all naturally derived bioinks are compatible with inkjet- and extrusion-based bioprinters but require chemical modification in order to be compatible with DLP- and TPP-based bioprinters. A list of commonly used naturally derived biomaterials and their uses can be found in Table 75.2.

Synthetically derived biomaterials

Synthetically derived biomaterials are often used for 3D tissue fabrication as they can be consistently produced and allow for easy control over mechanical properties, degradation rate, and printability. These materials can also be easily combined with nanoparticles in order to create functionalized scaffolds such as detoxification devices [113]. In addition, the backbones of these synthetic materials can be chemically modified to include cell-binding moieties such as RGD and YISGR in order to improve biocompatibility and cellular integration. A few of the most commonly used, synthetically derived biomaterials are poly(ethylene glycol) (PEG), Pluronic F-127, and poly(ε-caprolactone) (PCL). These materials are most commonly used as scaffolding materials due to their ease of printability and robust mechanical strength compared to naturally derived biomaterials.

PEG-based bioinks are among the most common hydrogels used in biomedical applications as they are nontoxic, nonimmunogenic, and have easily tunable mechanical properties via molecular weight modification. PEG alone has fairly poor mechanical properties, however, chemical addition of PEG diacrylate (PEGDA) or PEG methacrylate (PEGMA) groups facilitate photocrosslinking when in the presence of a photoinitiator, which results in increased mechanical strength. Therefore PEGDA and PEGMA are the most common forms of PEG used in 3D biofabrication. The addition of photopolymerizable groups allows the mechanical properties of PEG to be further tuned based on the duration and intensity of UV light exposure [103] wherein longer duration and higher light intensity increases stiffness. Due to its easy printability, fast photopolymerization (seconds), and relatively low cost, PEGDA and PEGMA are widely used in inkjet-, extrusion-, DLP-, and TPP-based printing. Common applications of PEGDA and PEGMA include cell encapsulation [114,115], biomimetic scaffold fabrication [102,103,107,108,116–118], and microfluidic devices [119–121].

Pluronic F-127 is a nontoxic poloxamer compound that undergoes reverse polymerization—increasing cross-linking with increasing temperature—and is compatible with extrusion-based bioprinting. Pluronic F-127 is also compatible with DLP- and TPP-based bioprinting after chemical modification [122,123] or combination with other photopolymerizable materials [124,125]. The temperature of cross-linking varies between 10°C and 40°C, depending on the molar mass, percentage of composites, and functionality [124,126]. This requires heating systems to be in place during the fabrication process, which, in combination with its relatively high viscosity, have precluded its use with inkjet-based bioprinters [36]. Pluronic F-127 has been used to encapsulate cells, although it typically degrades fairly quickly (within hours), making it more useful for cell delivery than as a long-term scaffold [127]. The rapid degradation time and reversible polymerization characteristics of Pluronic F-127 make it an attractive biomaterial for drug delivery and controlled release applications [128], as well as serving as a sacrificial bioink. Pluronic F-127 can be printed during the initial 3D fabrication process, only to be washed out after the construct acquires enough rigidity to retain shape [123,129–131]. This allows for the relatively simple construction of conduits or voids within a construct.

PCL is a thermoplastic commonly used with inkjet- and extrusion-based bioprinting platforms. PCL has a

TABLE 75.2 List of natural polymers commonly used for biofabrication.

	Collagen	Alginate	Hyaluronic acid	Matrigel
Printability	Moderate	Easy	Moderate	Moderate
Printer compatibility	Inkjet, extrusion, DLP	Inkjet, extrusion, DLP	Inkjet, extrusion, DLP, TPP	Extrusion
Cross-linking method	Thermo, pH, photo	Ionic (Ca^{2+}), photo	Chemical, photo	Thermo
Cross-linking speed	Seconds–1 h	Seconds	5–30 min	20 min–1 h
Chemical modification for photopolymerization (refs)	Methacrylation [63–65]	Methacrylation [66]	Methacrylation [67,68] Thiol(-ene) [60,69]	N/A
Advantages	Promotes cell adhesion and expansion, strong in vitro/in vivo biocompatibility, nonimmunogenic	High biocompatibility, cheap, nonimmunogenic, fast cross-linking time, generally low viscosity	Enhanced chondrogenesis/ osteogenesis, biocompatible, biodegradable, high solubility	Contains growth factors, facilitates cell growth/ adhesion
Disadvantages	Slow cross-linking time (0.5–1 h), nonhomogeneous cells distribution	Poor cell adhesion (no natural cell-adhesive moieties), slow degradation	Must be chemically modified, poor mechanical properties, slow cross-linking	Poor mechanical properties, must be combined with other materials
Common applications	Cell encapsulation, functionalize material surfaces	Composite bioinks, cell encapsulation, tubes, strands	Cell encapsulation, cell delivery, drug delivery, wound healing	Basement membrane, increase biocompatibility, promote cell growth
References	[27,47,70–74]	[75–85]	[60,68,69,86–90]	[91–93]

	Agarose	Fibrin	Decellularized ECM	Gelatin methacrylate
Printability	Easy	Easy	Moderate	Easy
Printer compatibility	Inkjet, extrusion	Inkjet, extrusion	Inkjet, extrusion, DLP	Inkjet, extrusion, DLP, TPP
Cross-linking method	Thermo	Fibrinogen + thrombin	Thermo	Thermo, photo
Cross-linking speed	Seconds–minutes	Seconds	Minutes	Seconds
Chemical modification for photopolymerization (refs)	N/A	N/A	Mix w/other photopolymerizable materials	Methacrylation
Advantages	Fast polymerization, thermally reversible cross-linking	Fast polymerization, highly adhesive, cell adherent, binds to growth factors	Contains native growth factors of target tissue, high cell adherence, high cell proliferation	Retains cell-binding motifs, fast polymerization, good cell viability, tunable mechanical properties
Disadvantages	No cell adherence proteins, poor mechanical properties tunable by concentration	Poor mechanical properties, difficult to control geometry	Poor mechanical properties, batch-to-batch variation, slow cross-linking time	Not as durable as synthetic polymers
Common applications	Cell encapsulation, structural support/ scaffolding	Adhesive, cell encapsulation, cell delivery	Incorporation with other materials for tissue-specific cellular response	Widely used, cell encapsulation, increasing biocompatibility
References	[91,94–98]	[60,99–101]	[102–106]	[107–112]

DLP, Digital light processing; *ECM*, extracellular matrix; *TPP*, two-photon polymerization.

relatively low melting temperature of 60°C [132] and is commonly used as a primary structural component in scaffolds [129]. PCL does not contain any natural peptide sequence motifs, so it is largely used in conjunction with other naturally derived polymers or functionalized materials to create composite structures [133,134]. As PCL is relatively stiff compared to other synthetic polymers, it is typically used in cartilage and bone tissue engineering [135,136].

Cell selection

A critical step in the design of 3D tissue models is the appropriate selection of cell sources as this directly influences the performance as well as accuracy and relevancy of the resulting model. Moreover, the cells chosen for the model must be able to replicate the key physiological characteristics of the normal or pathological states for the tissue of interest. Given these criteria, there are several cell sources available that can be classified as either primary cells, cell lines, or stem cell–derived cells. In the context of tissue biofabrication, it is also important that the cell source selected is capable of ex vivo expansion to meet the requirement of high cell numbers for bioprinting often ranging between 1×10^6 and 1×10^8 cells/mL depending on the application [137]. Reproducibility and consistency of the cells produced in terms of maintaining the desired phenotype and function are equally important postexpansion as well as during in vitro culture in a 3D format post fabrication. Other factors to consider also include the ability for the cells to survive the physical stressors brought upon during the bioprinting process as well as continued self-renewal thereafter to remodel the tissue construct and maintain appropriate cellular density in long-term culture.

Primary cells are terminally differentiated and have the quality of being a direct representation of the phenotype, maturation state, and functional features of the native tissue functional unit [138]. These cells are harvested from patient biopsies and have been reported to be used successfully for generating bioprinted liver, cartilage, and skin tissues [139–141]. However, because these cells need to be isolated, a major drawback is their limited availability as small samples can only be harvested at a time to avoid donor site morbidity and the procedures performed can be complex and invasive [142]. Furthermore, primary cell culture usage is further complicated by their limited capability for ex vivo culture as these cell types often lack in vitro proliferative capacity, are sensitive to culture conditions, prone to dedifferentiation, and susceptible to changes in phenotype expression over prolonged culture [142]. For the generation of reproducible tissue models, inherent donor-to-donor variability and risk of obtaining cells in a diseased state also poses a challenge for primary cells as this will affect the consistency in the functional response of the models.

To address the issues regarding primary cells, cell lines are a convenient substitute as they have been altered via viral transfection to be able to proliferate in vitro indefinitely by preventing normal cellular senescence, thus enabling large populations to be cultivated. To date, a plethora of cell lines have been established for several tissues including the immortalized normal adult kidney cell line human kidney−2 cells, human liver carcinoma cell line HepG2, human cervical cancer HeLa, mouse cardiac muscle cell line HL-1, and mouse fibroblast cell line 3T3 cells. While cell lines serve as important biological tools by offering several advantages such as ease of use, reproducibility, cost-effectiveness, unlimited supply, and circumventing ethical concerns regarding human and animal tissue use, it is important to recognize that although cell lines display functional features of primary cells they are not identical in behavior [143]. This is because cell lines have been genetically manipulated and serial passaging can result in variations of their native genotypic and phenotypic expression profiles, in addition to changes in response to different stimuli [143]. For example, it was found in a comparative study that using the human hepatoma cell lines, HepG2 and HepaRG cells, exhibited reduced sensitivity to drug toxicity compounds compared to primary human hepatocytes [144]. As such, these idealized cell types provide limited predictive value as they have poor resemblance to native primary cells, and it is imperative that the data collected from in vitro models produced from cell lines to be interpreted with caution.

More recently, the use of stem cell–derived cells originating from embryonic stem cells, pluripotent stem cells, or adult stem cells has gained popularity as a viable source due to their self-renewing properties and differentiation capacity into various functional cell types. Embryonic stem cells are totipotent thus enabling them to differentiate into specialized cell lineages derived from any of the three embryonic germ layers [145]. For instance, this includes plasticity to differentiate into cells of cardiac, neuronal, as well as hematopoietic origins. Embryonic stem cells can be cultured continuously for long periods of time in the undifferentiated state and are practical for generating large populations, thus enabling their potentiality to provide an unlimited number of any specialized cell type [145]. However, with ethical concerns regarding the use of human embryos in addition to risks of teratoma formation and allogenic origin, these factors may hinder their application for cell-based regenerative therapies [146]. In the last decade, techniques to generate iPSCs pioneered by Takahashi and Yamanaka resulted in a paradigm shift in the cell source landscape [147]. By introducing the four factors *Klf4*, *Sox2*, *Oct3/4*, and *Myc* this method enabled researchers to reprogram adult cells into iPSCs that

were capable of pluripotent differentiation and possess characteristics similar to embryonic stem cells with extensive self-renewal capacity [147]. Currently, a large range of protocols have been developed to produce specialized cell types from easily accessible human fibroblast, adipose-derived stem cell (ADSC), or peripheral blood cell sources into hepatocytes, neural stem cells (NSCs), pancreatic cells, and gastric epithelial cells [148]. The use of iPSCs also garners a significant advantage by not only overcoming the ethical concerns associated with embryonic stem cells but also enabling the possibility of patient-specific cell therapy for clinical applications. More specifically, cells can be obtained from patients to produce patient-specific tissue models for developing personalized medicine for the treatment of various rare diseases and elucidation of their associated pathological mechanisms. Finally, adult stem cells which can be autologously or allogeneically harvested from adult tissues such as adipose [149], bone [150], umbilical cord blood [151], and skin [152] have shown promise as a viable regenerative cell source in recent years. Unlike embryonic stem cells and iPSCs, adult stem cell populations are multipotent rendering them capable of differentiating into two or more cell types but are more limited in lineage plasticity. For instance, cells such as ADSCs and bone marrow–derived stem cells have been demonstrated to successfully differentiate toward the adipogenic, chondrogenic, and osteogenic lineages in vitro [153,154]. Although promising as an accessible cell source, adult stem cells are still met with challenges in terms of variability in self-renewal capacity, differentiation potency, donor-to-donor differences, as well as the steps taken in the isolation, identification, and purification of the multipotent stem cell population from the stroma [155]. As a result, many of these factors pose the same issues as primary cells by affecting their reproducibility and predictive capability which may compromise the quality of the final tissue model.

Three-dimensional tissue models for drug screening, disease modeling, therapeutics, and toxicology

In the following sections the application of 3D biofabricated constructs for in vitro disease modeling, high throughput drug testing platforms, and in vivo therapeutics will be discussed. Specifically, the implementation of various biofabrication strategies to recapitulate both normal and diseased brain, nerve, cancer, heart, liver, and vascularized tissue will be reviewed.

Brain and nerve tissue models

The brain is the most complex tissue in the body, consisting of numerous different cell types organized into intricate layers and compartments. Understanding the development, structure, and degenerative disorders which affect the brain is the main goal of neurobiology. Due to practical and ethical reasons, studying neural structure, development, and disease has been largely reliant on animal studies consisting of mainly rodent models. Brain structure and development vary greatly between animal and human models and thus has been a large motivation for the development of in vitro models of the human brain.

3D bioprinting neural structures present a number of challenges that are unique to accurately replicate brain structure and organization. This may partially contribute to the lack of successful 3D fabricated models in brain tissue compared to other more simplistic tissues. The human brain consists of roughly 86 billion neurons and 85 billion nonneuronal cells [156]. The nonneuronal cells, referred to as glial cells, include astrocytes, microglia, oligodendrocytes, endothelial cells, and pericytes [157]. Glial cells perform a wide spectrum of functions throughout the brain in order to maintain normal neuronal function. Damage to or imbalance of these cells leads to neurodegenerative disorders in vivo. Therefore in order to develop accurate in vitro models, the precise number and distribution of multiple cell types must be simultaneously accounted for, which is challenging but not impossible with 3D bioprinting.

The capacity to differentiate into multiple cell types and the self-renewal capacity of NSCs have made them attractive candidates for generating models of brain tissue. Using an extrusion-based printer, Gu et al. printed human NSCs differentiated in situ into a complex structure consisting of neurons, as well as astrocytes and oligodendrocytes [158]. Mature-fabricated models contained rounded soma and extensive neurite outgrowth, similar to native neuronal morphology. These models also exhibited bicuculline-induced increased calcium response, thus demonstrating that the differentiated neurons had formed into functional GABA receptors [159]. Hsieh et al. also utilized extrusion-based bioprinting to develop a unique polyurethane bioink containing NSC. When implanted into a zebrafish model of traumatic brain injury, the zebrafish recovered normal swimming ability and experienced reduced mortality [160].

Overall, 3D bioprinting brain tissue is still in its infancy compared to other methods of in vitro brain modeling. The most complex models of the brain are cerebral organoids. Cerebral organoids are a scaffold-free, human iPSC–derived “mini-brain” model, which contains various discrete, interdependent regions, fabricated using a hanging drop culture environment [161]. The culturing conditions promote self-organization and self-patterning of iPSCs into various brain regions without the use of exogenous patterning factors. Recently, these organoids

have been combined with poly (lactic-co-glycolic acid) (PLGA) microfilaments to enhance neuroectoderm formation and form a distinct cortical plate, which is considered the final step in corticogenesis [162]. These microfilament-engineered cerebral organoids formed distinct layers and had excellent neuronal organization compared to cerebral organoids alone (Fig. 75.2). A limitation of these models is that they do not contain microvasculature, which precludes the study of the blood–brain barrier (BBB). The BBB is a highly selective semipermeable membrane that regulates ion, molecule, and cell transfer from vasculature to the brain, and alterations in its function are involved in pathology and progression of disease [163]. To develop an in vitro model of the BBB, Nzou et al. utilized a hanging drop culture environment to develop an organoid model comprised of the six constituent cell types found within the cortex of the brain [164]. The resulting spheroids are capable of junction formation and selective permeation of ions, making this model attractive for testing a drug candidate's ability to cross the BBB.

Nerve injuries can be grossly classified into two general categories: (1) peripheral nerve injuries and (2) spinal cord injuries. In particular, peripheral nerve injuries

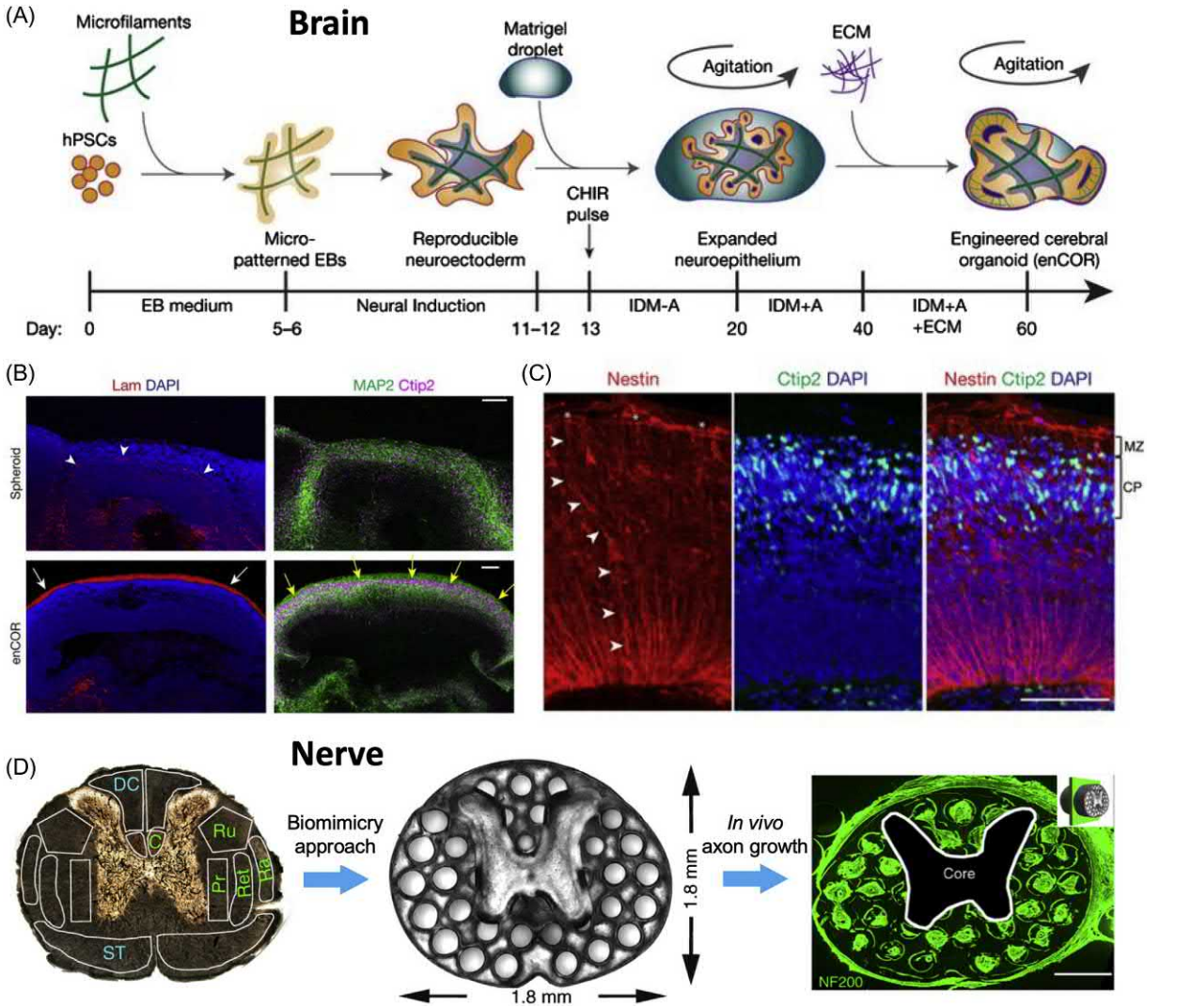

FIGURE 75.2 Brain and nerve tissue models. (A) Schematic of the method for generating the engineered cerebral organoids. (B) Immunohistochemical staining for laminin and the neuronal markers MAP2 and Ctip2 in engineered cerebral organoids. The organization of laminin, MAP2, and Ctip2 in the cerebral organoid containing microfilaments (bottom) is typical of the CP in vivo. (C) Nestin staining reveals long radial glia (*arrowhead*) organization that terminates outside the CP and MZ at the outside of the organoid. (D) *Left*: Axial slice of a spinal cord depicting the regions of white and gray matter. *Center*: 3D bioprinted scaffold with physiologically informed geometry. Scaffold was printed using a DLP-based bioprinter in 1.6 seconds. *Right*: A cross section through an implanted scaffold, 4 weeks after implantation. Green indicates axons. Scale bar = 200 μm. *3D*, Three-dimensional; *CP*, cortical plate; *DLP*, digital light processing; *MZ*, marginal zone. *Reproduced with permission from (A–C) Springer Nature (2017) [162] and (D) Springer Nature (2019) [118].*

require approximately 200,000 surgeries annually in the United States [165,166]. Current clinical approaches to healing nerve injuries involve trying to suture the proximal and distal ends of the injury (<5 mm gap), or through autologous or cadaveric nerve transposition [167,168]. While autografts are the current gold standard, they require sacrificing nerve tissue from another part of the body and the resulting nerve may not actually fit the injury site [165]. This has motivated the development of peripheral nerve guides, to try to bridge the gap in a peripheral nerve injury [167,168]. Zhu et al. utilized a DLP-based 3D bioprinter to fabricate peripheral nerve guides with anatomically informed geometry [117]. In a rat injury model of peripheral nerve injury (sciatic transection) they were able to demonstrate improved functionality of motor and sensory function, as well as histological verification of nerve growth into the scaffold. Spinal cord injuries affect an estimated 285,000 people in the United States and have poorer outcomes and fewer surgical procedures available than peripheral nerve injuries [169]. Koffler et al. expanded upon the prior study and fabricated nerve guides for spinal cord injury repair using the same DLP-based 3D bioprinter [118]. The nerve guide geometry was informed by the anatomical structure of the spinal cord, and additionally included "neural relays" consisting of neural progenitor cells integrated into the scaffold (Fig. 75.2). In a rat spinal cord injury model (T3 spinal cord transection), implanted scaffolds were found to integrate with native injured host axons and significantly improve functional outcomes. Importantly, both of these studies demonstrated the capability to print human-sized scaffolds, as well as scaffolds with geometry extracted from MRI images. These patient-specific scaffolds hold promise for a future clinical translation of 3D bioprinting.

While 3D fabrication strategies for nerve are promising, complex models of 3D fabricated brain tissue still need to be developed. Brain tissue contains such a vast array of cells and has a unique microenvironment compared to the rest of the body. New biofabrication strategies must therefore focus on recapitulating the layered, complex organization of neural tissue in models that are capable of being handled.

Cancer models

Over 18 million new cases of cancer and over 9 million cancer-related deaths were estimated to have occurred in 2018 [170]. Despite its prevalence, the lack of understanding of tumorigenesis and metastases affects the ability to develop successful drugs to fight cancer. Currently, the percentage of cancer drugs that transition into successful clinical therapeutics is about 8% [171]. This is partially due to the lack of suitable in vitro and animal models for studying the complex tumor microenvironment (TME) in humans. While conventional 2D cancer models have yielded some promising results [172], they inadequately mimic the 3D TME [173,174]. This has given rise to the use of 3D bioprinting as a technique to fabricate in vitro cancer models to study tumor proliferation, metastasis, and drug response.

TME is highly complex, heterogeneous, and disease dependent, affecting cell proliferation and tumor characteristics. Zhao et al. utilized 3D extrusion-based printing to investigate differences in HeLa cell proliferation, matrix metalloproteinase (MMP) production, and chemoresistance in 2D planar culture versus 3D tumor modeling [175]. HeLa cells were found to have higher proliferation and formed spheroids in 3D compared to forming a monolayer in 2D culture. In addition, HeLa cells in 3D had higher MMP production and were more resistant to paclitaxel, a commonly used chemotherapy drug. Swaminathan et al. expanded upon this work to demonstrate that preformed spheroids prior to printing, demonstrated greater resistance to paclitaxel than individually printed cells in a breast cancer model [176]. The findings from these studies suggest that more biomimetic cell−cell and cell−matrix interactions may contribute to the differences in cancer cell behavior and function in 2D versus 3D microenvironments.

Tumor metastasis leads to 90% of cancer death and decreases 5-year survival rates. Therefore there has been much interest in evaluating how TME affects tumor metastasis in vitro. Using a DLP-based 3D printing platform, Soman et al. fabricated a scaffold with a log-pile architecture with tunable material properties [177]. The differences in cell migration in normal and TWIST oncogene−transformed breast endothelial cells were evaluated in 2D and 3D in scaffolds with soft or stiff substrates. They found that while no differences in cell migration were found in 2D between soft and stiff substrates, substantial differences were found between soft and stiff substrates for the cancer cells in 3D. This is important as it demonstrates that observations conducted in a 2D system cannot be directly extrapolated to 3D. Huang et al. further evaluated cancer metastasis utilizing a DLP-based bioprinter by fabricating a biomimetic microstructure with vasculature informed channel sizes (Fig. 75.3) [121]. The migration behavior of normal fibroblast (10T1/2) and HeLa cells was evaluated in channels with 25, 45, and 120 μm width. Channel width had no effect on average migratory speed of fibroblasts, but HeLa cell speed was found to significantly decrease with increasing channel width (Fig. 75.3). This suggests that the different responses of normal and cancerous cells to different geometric environments could be used as a tool to screen cancerous cells. These studies demonstrate how the 3D environment can drastically change the behavior

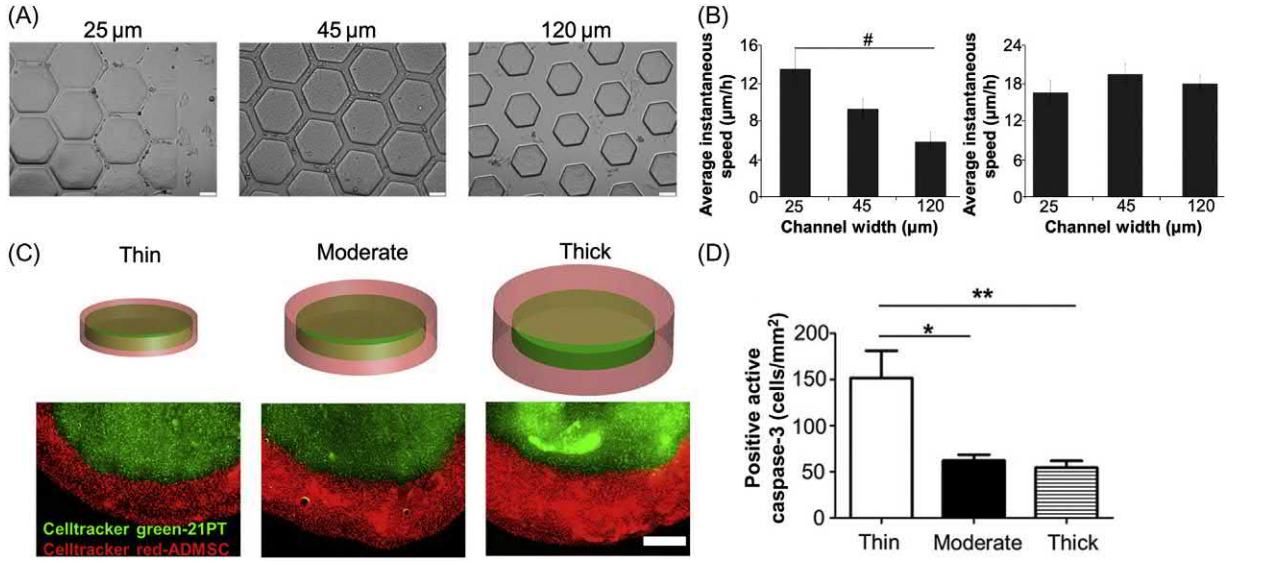

FIGURE 75.3 Cancer tissue models. (A) Optical microscope images of HeLa cells seeded on fabricated PEGDA microstructures. Scale bar = 100 μm. (B) Average instantaneous speed of HeLa cells (left) and 10T1/2 cells (right) cultured on microstructures. Channel width had a significant effect on instantaneous cell speed for cancer cells but not fibroblasts. (C) Bioprinted constructs consisting of primary breast cancer cells (21PT; *green*) and ADMSCs (*red*). Constructs were fabricated with thin, moderate, and thick layers of ADMSCs around the 21PT printed disk. Scale bar = 250 μm. (D) Density of Caspase-3 positive (apoptotic) 21PT cells in constructs with thin, moderate, and thick layers of ADMSCs after treatment with doxorubicin (chemotherapy drug). Increasing the amount of ADMSCs around 21PT cells decreases the sensitivity of 21PT cells to doxorubicin. *ADMSCs*, Adipose-derive mesenchymal stem/stromal cells; *PEGDA*, poly(ethylene glycol) diacrylate. *(A and B) Reproduced with permission from Springer Science + Business Media (2014) [121]; (C and D) reprinted (adapted) with permission from Heinrich MA, Bansal R, Lammers T, Zhang YS, Michel Schiffelers R, Prakash J. 3D-bioprinted mini-brain: a glioblastoma model to study cellular interactions and therapeutics. Adv Mater 2019;31:e1806590. ©2018 American Chemical Society [179].*

of cancer cell mobility and can be used to inform future studies investigating tumor metastasis.

Stroma tumor interactions have been increasingly identified as a key factor in treatment response to various drugs. In fact, stroma-induced drug resistance and stroma-induced synthetic lethality are linked to the TME with which tumor cells interact [178]. Wang et al. demonstrated that the addition of a layer of stromal cells around a disk of bioprinted breast cancer models decreased the breast cancer cell's sensitivity to a chemotherapy drug (doxorubicin) (Fig. 75.3) [179]. Further, the thicker the layer of stromal cells, the less breast cancer cell apoptosis was observed. This printing system consisted of multiple syringe extrusion-based printer, separate bioinks for each cell type, each encapsulated in a combination of methacrylated gelatin, hyaluronic acid, and gelatin. During the extrusion process, the construct had to be photo-crosslinked three separate times in order to fabricate a stable structure. Heinrich et al. recently utilized a similar setup in order to investigate the relationship between glioblastoma-associated macrophages and glioblastoma multiforme [180]. They printed a "mini-brain" consisting of a glioblastoma tumor surrounded by a large number of macrophages. They demonstrated that glioblastoma cells actively recruit macrophages and polarize them to have a more migratory phenotype. In addition, tumors cocultured with macrophages were found to have a much higher growth rate than tumors cultured alone. These studies demonstrate that the inclusion of stromal cells into the TME enhances clinical relevance of the models in vitro.

3D bioprinting of cancer models has the potential to provide insight into how the TME affects tumor development, behavior, metastasis, and invasion. Future works focusing on patient-specific cells have the potential to provide more insight on patient-specific as well as stage-specific behavior of various types of cancer. Further, 3D bioprinting patient-specific in vitro tumor models has the tremendous potential to screen drugs which may or may not be effective, potentially resolving the disease faster and improving patient outcomes. However, additional work is needed to develop the materials and cell sources in order to create repeatable, physiologically relevant tumor models.

Heart tissue models

Worldwide, cardiovascular diseases (CVD) are a major cause of morbidity and mortality [181]. Myocardial infarction (MI) and valvular heart disease (VHD) are some of the main forms of CVD. In the United States, an estimated 720,000 people experience a new MI with 335,000 having a recurrent MI annually [181] and an

estimated 2.5% of the population are thought to have VHD [182]. As cardiac muscle has limited capacity for self-repair, current treatment strategies include grafting diseased tissues or inserting artificial prostheses to attempt to restore function [183]. However, each of these approaches is complicated by their own respective disadvantages which include lack of donor tissue, immune rejection, anticoagulation therapy, and limited durability [184]. These factors have driven a large amount of resources to develop predictive preclinical platforms for cardiac drug testing, as well as durable patient-specific therapies to treat CVD and physical models for presurgical planning [185,186].

Current models of 3D cardiac tissue focus around seeding cells atop, or encapsulating cells within a hydrogel-based scaffold [52,187], then utilizing 3D aligned cultures [188], mechanical stretching [189], or electrical pacing [189,190] to promote mature cardiomyocyte phenotypes. However, by using 3D printing, it is possible to achieve mature aligned cardiomyocytes without any external stimulus. For instance, Liu et al. employed a DLP-based printer to directly print human iPSC–derived cardiomyocytes into aligned patterned slabs suspended between two pillars [191]. While both slabs and aligned cardiomyocytes exhibited spontaneous synchronous beating, cardiomyocytes printed in slabs beat without directional preference, whereas aligned cardiomyocytes beat along the direction of the patterning (Fig. 75.4). Furthermore, the deflection of pillar structures in which the cardiomyocytes were printed onto enabled analysis of cardiac force generation and drug response in a high throughput manner [116]. These studies demonstrate the ability to rapidly fabricate in vitro cardiac models, capable of supporting rapid drug testing. Some drugs may disproportionately benefit or harm certain genotypes, ethnicities, sexes, and ages [192]. Since the cardiomyocytes used in this platform are derived from human iPSCs, this allows for in vitro drug testing for all potential patient populations.

During MI, reduced blood flow to the heart results in ischemia and subsequently leads to large necrotic patches of heart tissue. TE cardiac patches are designed to mimic the native ECM and provide mechanical support as well as delivery of cells to the area of MI. For example, Jang et al. utilized a dual nozzle, extrusion-based printer to fabricate a prevascularized stem cell patch [193]. Both cardiac progenitor cells (CPCs) and mesenchymal stem cells (MSCs) were printed in a dECM bioink containing vascular endothelial growth factor (VEGF), and cultured for 5 days. Using a standardized model of MI in a rat, implants were surgically implanted before sacrifice at up to 56 days' postinjury. Patterned patches were found to increase ejection fraction and decrease fibrosis compared to patches consisting of simply mixed CPCs and MSCs.

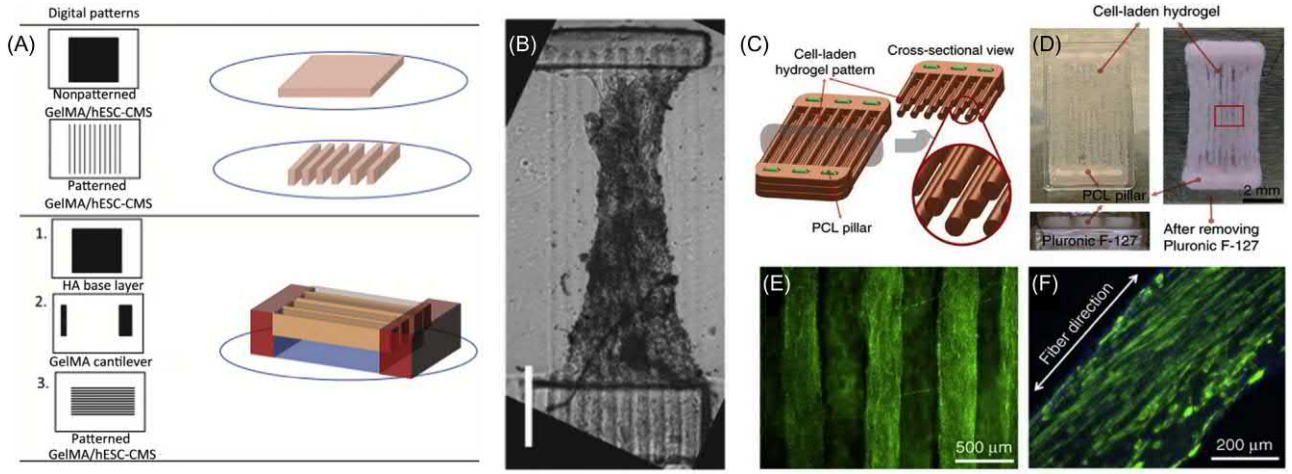

FIGURE 75.4 Heart tissue models. (A) *Top:* Two sets of digital patterns including a slab and aligned patterns for encapsulated cell printing with a DLP-based bioprinter. *Bottom:* The masks for the multilayer pattern to produce 3D scaffolds. First, a hyaluronic acid base layer is printed; second, a gelatin methacrylate cantilever system; and third, parallel lines of hESC-CMs. (B) Optical image of the resulting patterned hESC-CMs printed in the force gauge scaffold after culture for 21 days. Scale bar = 500 μm. (C) Designed fiber bundle structure for muscle organization. PCL pillars (*green*) were used to maintain the structure and to induce the compaction phenomenon for cell alignment. (D) 3D patterning outcome of designed muscle organization (left) before and (after) removing the sacrificial material (Pluronic F-127). The printed construct was cross-linked with thrombin solution to induce gelation of fibrinogen and the uncross-linked sacrificial material was removed by dissolving with cold medium. (E) The Live/Dead staining of the encapsulated cells in the fiber structure indicates high cell viability after the printing process (*green*: live cells; *red*: dead cells). (F) Immunofluorescent staining for myosin heavy chain of the 3D printed muscle organization after 7 days of differentiation. The encapsulated myoblasts aligned along the longitudinal direction of the fiber structure. *3D*, Three-dimensional; *DLP*, digital light processing; *hESC-CM*, human embryonic stem cell–derived cardiomyocytes; *PCL*, poly(ε-caprolactone). *Reproduced with permission from (A and B) Elsevier (2019) [191] and (C–F) Springer Nature (2016) [129].*

Furthermore, revascularization of the damaged myocardium was observed. These results demonstrate that an organized 3D bioprinted scaffold can not only improve cardiac function via mechanical support but also promote neovascularization of the damaged tissue.

Cardiac valves play a major role in the proper functioning of the cardiovascular system by ensuring proper blood flow through the four chambers of the heart. In severe cases of VHD, valves must be surgically replaced. There is increased interest in developing a TE heart valve rather than relying on mechanical prosthetics, donor supplied allografts, or porcine/bovine xenografts. Since cardiac valves have highly specific geometry integral to proper function, 3D bioprinting has been sought as an attractive fabrication method. Hockaday et al. used an extrusion-based 3D bioprinter to fabricate a biocompatible heterogeneous valve with flexible leaflets and a rigid root [194]. Duan et al. implemented a similar model using extrusion-based 3D bioprinting and incorporated valvular interstitial cells and smooth muscle cells encapsulated in a combination of methacrylated hyaluronic acid and methacrylated gelatin rather than a synthetic polymer [195]. Further work by van der Valk et al. sought to use a similar approach by encapsulating valvular interstitial cells in order to develop an in vitro model of calcific aortic valve disease (CAVD), the most prevalent form of VHD [196]. Microdissection and careful mechanical testing of a cadaveric human ventricle with CAVD was performed to accurately model the disease progression. Mechanical properties of the fabricated construct were varied by modulating the ratios of the main constituents of their bioink [gelatin methacrylate (GelMA) and methacrylated hyaluronic acid]. The resulting multilayered 3D construct recapitulated the layer-specific mechanical properties of the dissected valve and provided a novel 3D model for studying CAVD.

Overall, 3D bioprinting is a promising technique for the fabrication of in vitro cardiac models to in vivo cardiac therapies. A common thread between successful platforms is the importance of cellular alignment and mechanical properties of the scaffolds, which are integral features of the heart architecture. These features are not only integral factors to consider in cardiac muscle but in skeletal muscle as well. 3D fabrication of skeletal muscle models for in vitro disease modeling and in vivo muscle repair consistently demonstrates that fiber alignment during the initial formation of a scaffold leads to organized muscle tissue with superior functional outcomes (Fig. 75.4) [129,197]. In tissues such as cardiac and skeletal muscle, where aligned cellular organization is essential for normal tissue function, 3D printing is a key tool for fabricating physiologically accurate tissues.

Liver tissue models

Liver is a densely populated organ composed of approximately 120 million cells/gram in humans and is made up of mainly hepatocytes as well as several tissue-specific cell types including Kupffer cells, epithelial cells, stromal cells, and sinusoidal endothelial cells [198]. These cells are arranged into highly organized hexagonal hepatic lobule functional units. Within each hepatic lobule, hepatocytes are situated into plates that radiate in an outward direction from the central vein with portal triads located at each vertex composed of the hepatic arteriole, portal venule, and bile duct. Venous blood and arterial blood enter through the portal vein and hepatic artery, respectively, and flow through the sinusoidal regions and drain into the central vein of each hepatic lobule.

Since liver is a major site for drug adsorption, distribution, metabolism, and excretion there is a critical need for novel liver tissue models as tools to more accurately predict drug metabolism, pharmacokinetics, and hepatotoxicity for the development of potential new therapies. Namely, this demand has largely driven strong motivation in the pharmaceutical industry for the development of better liver models for drug-induced liver injury (DILI) as this is a major cause for drug failure resulting in liver transplantation or morbidity in patients [199]. To address this issue, it was recently shown that a 3D bioprinted liver tissue using patient-derived primary hepatocytes and nonparenchymal endothelial and hepatic stellate cells were suitable as a multicellular model for DILI [139]. Using a scaffold-free assembly process of cellular spheroids, Nguyen et al. patterned the different cells types using an extrusion-based bioprinter into a microarchitecture composed of several compartments filled with hepatocytes and surrounded by nonparenchymal cells (Fig. 75.5). Using this bioprinted liver, they demonstrated that the printed tissue was capable of maintaining long-term function over 4 weeks in culture including albumin secretion and expression of drug-induced enzyme activities of cytochrome P450s [139]. In particular, it was shown that model was sensitive to trovafloxacin dose-dependent toxicity at clinically relevant doses, which is normally undetected in standard in vitro systems [200]. Similarly, Ma et al. developed a complex heterogeneous liver tissue model that uniquely replicates the physiological microarchitecture and microscale features of the hexagonal lobule unit for drug screening [108]. In this work, a DLP-based 3D bioprinter was employed to pattern human iPSC–derived hepatic progenitor cells (iPSC-HPCs) within a GelMA matrix, as well as human umbilical vein endothelial cells (HUVECs) combined with ADSCs in a GelMA and glycidyl methacrylate hyaluronic acid (GMHA) matrix to serve as the supporting cell population to form a triculture 3D liver model as shown in Fig. 75.5.

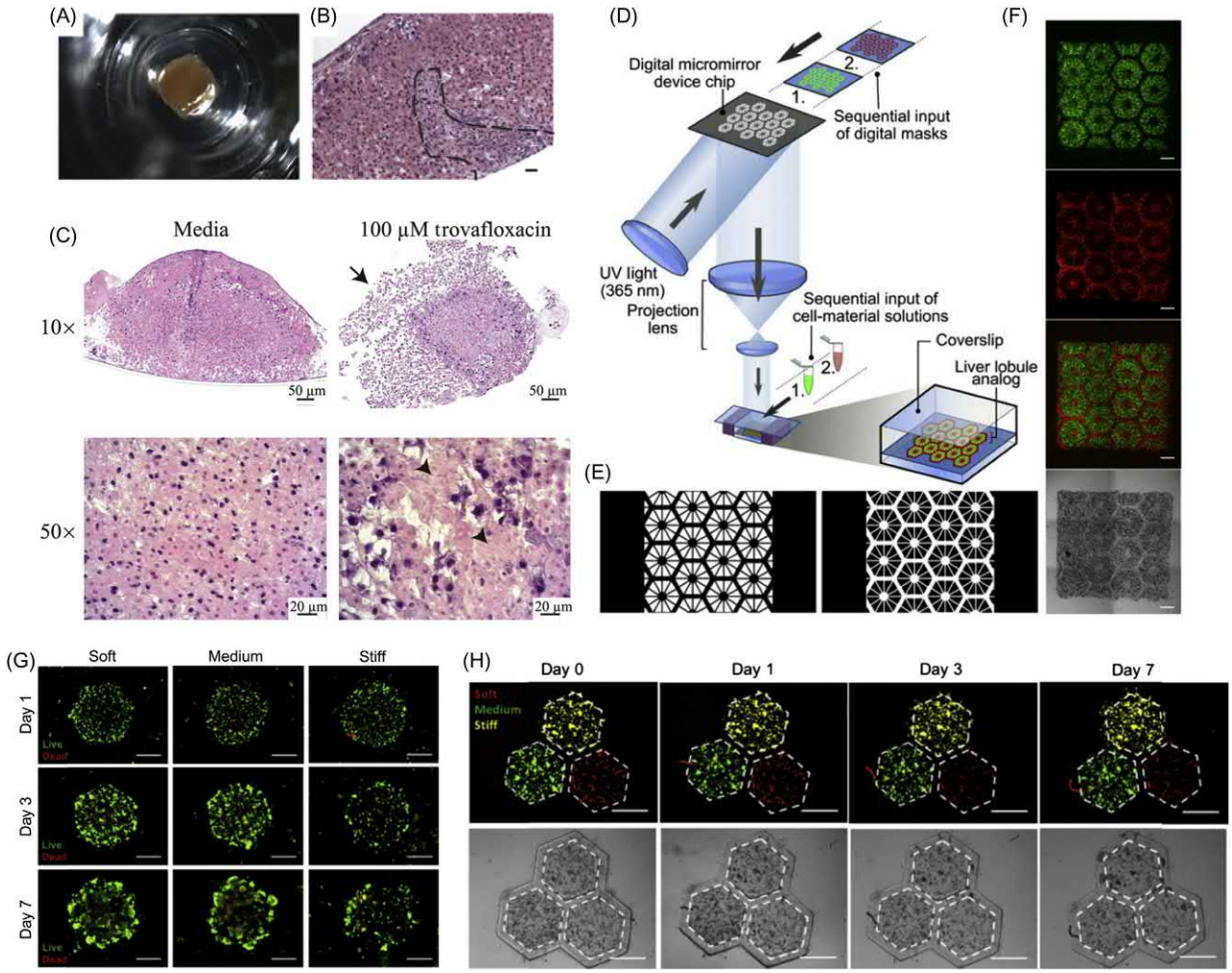

FIGURE 75.5 Liver tissue models. (A) Scaffold-free spheroid-based bioprinted liver tissue and (B) representative H&E image showing compartmentalization of the hepatocytes and nonparenchymal cell populations indicated by the dashed line. Scale bar = 25 μm. (C) Images of H&E stained bioprinted liver tissues with and without trovafloxacin treatment. (D) Schematic of a DLP-based 3D bioprinter, (E) hexagonally shaped digital patterns used to fabricate the liver tissue model, and (F) resulting fluorescent images of the printed liver tissue showing iPSC-HPCs (*green*) surrounded by supportive cells (*red*). Scale bar = 500 μm. (G) Representative Live/Dead images of HepG2 cells encapsulated in varying stiffnesses of liver dECM−GelMA hydrogels. Scale bar = 500 μm. (H) Images of CellTracker stained HepG2 cells to visualize their invasion into surrounding stromal regions over time from soft (*red*), medium (*green*), and stiff (*yellow*) conditions. Scale bar = 500 μm. *3D*, Three-dimensional; *dECM*, decellularized extracellular matrix; *DLP*, digital light processing; *GelMA*, gelatin methacrylate; *iPSC-HPCs*, induced pluripotent stem cells−derived hepatic progenitor cells. *Reproduced with permission from (A−C) PLoS One (2016) [139]; (D−F) National Academy of Sciences, ©2016 [108]; and (G and H) Elsevier (2018) [103].*

Compared to 2D monolayer controls as well as iPSC-HPC only bioprinted liver constructs, Ma et al. demonstrated improved metabolic activity with regards to increased albumin and urea secretion over time in the triculture system over 19 days in culture. Maturation state also increased in the triculture system as characterized by a significantly higher expression of albumin (*ALB*), transthyretin (*TTR*), hepatocyte nuclear factor 4 alpha (*HNF4α*) along with a downregulation of the early marker alpha fetal protein (*AFP*) [108]. When treated with the hepatotoxic drug, rifampicin, CYP expression levels (i.e., *CYP2C9*, *CYP2C19*, and *CYP3A4*) significantly increased in the triculture model compared to untreated controls, while no observed significant increase was detected in the 2D monolayer and bioprinted iPSC-HPC only groups. These results suggest that the triculture model provided a suitable environment for drug induction potential of iPSC-HPCs in vitro.

The pathological mechanisms of liver fibrogenesis have also been largely studied as this condition develops over time in many types of chronic liver diseases and is a consequence of an imbalance in the reparative process following injury. As such, this results in changes in the liver tissue matrix due to an abnormal overproduction of

fibrillar collagen that disrupts normal liver microarchitecture, as well as the production of inflammatory cytokines and growth factors, which altogether deteriorates normal liver function [201]. Modeling liver fibrosis in vitro is challenging to recapitulate since the process in vivo is complex and involves multiple interactions between both resident cells as well as recruited cells. Since macrophages play an important role in liver fibrogenesis, Norona et al. aimed to further elucidate the role of Kupffer cells, which are specialized macrophages located in the liver sinusoids and involved in homeostatic functionality [202], following drug-induced fibrogenesis in a 3D bioprinted liver model [203]. Using a NovoGen Bioprinter platform (Organovo, San Diego, CA) a scaffold-free, two-compartment tissue geometry was produced comprising of hepatic stellate cells and endothelial cells within the border regions while primary human hepatocytes were positioned within the compartments in the presence or absence of Kupffer cells [203]. Over 28 days in culture, the liver tissues were treated with TGF-β1 and methotrexate to induce fibrotic injury. Interestingly, levels of lactate dehydrogenase activity, which was used as a general biomarker of cytotoxic response over the treatment period, was reduced in the liver tissue containing Kupffer cells for both TGF-β1 and methotrexate [203]. Furthermore, the release of miR-122, which was used as a specific hepatocyte injury biomarker, verified that the early onset of injury in response to TGF-β1 was delayed in the Kupffer cell containing model compared to the standard model without Kupffer cells [203]. On the other hand, miR-122 levels were also delayed in the methotrexate treated samples during initial exposure; however, the overall levels increased relative to the standard model across the treatment period. While both drugs resulted in similar biochemical responses, gene expression profiling further elucidated that Kupffer cells played an important role on baseline tissue function and the varied responses these cells have on different drug compounds [203]. Specifically, it was hypothesized that TGF-β1 acts after hepatocyte injury occurs during the intermediate stages of the response while methotrexate acts upstream by instigating hepatocyte injury prior to downstream fibrotic events [203]. In a different examination, a platform for studying the effects of pathologically relevant 3D matrix stiffness on hepatocellular carcinoma (HCC) progression and invasion was developed by Ma et al. (Fig. 75.5) [103]. Here, a 3D liver construct composed of decellularized porcine liver ECM and GelMA was developed such that the mechanical properties of the engineered liver tissue were precisely tuned to recapitulate varying stages of fibrotic liver conditions ranging from physiologically relevant softer than normal (0.5 kPa), normal (5 kPa), and cirrhotic (15 kPa) moduli [103]. Next, encapsulated HepG2 cells within the bioprinted scaffold were measured to evaluate their growth and invasiveness over 7 days. The results demonstrated characteristic restricted growth and upregulation of invasive markers of these cancer cells under cirrhotic stiffness compared to healthy stiffness controls, which confirmed the validity of the tissue model. This work affirms the feasibility of using 3D bioprinting to study HCC growth and invasion in a pathological mechanical environment.

Overall, these advanced engineered liver tissues highlight their potential use as more reliable, sensitive, and predictive models for improving the drug screening process. However, many of these 3D liver models are still in early development and limited in terms of their application in high throughput arrangements accustomed in the pharmaceutical industry. Moreover, to ensure the efficacy of future 3D liver models, they will need to satisfy the following criteria: (1) demonstrate significant advantage over conventional models such as 2D cultured hepatocytes, (2) exhibit long-term maintenance of normal function and phenotype as well as drug-metabolizing enzyme activities to enable prolonged in vitro hepatotoxicity testing, and (3) produce measurable outcomes in response to the development of DILI such as changes in mitochondrial function and the formation of reactive oxygen species [204].

Vascular tissue models

Adequate vascularization is critical for long-term survival by facilitating the delivery of oxygen and nutrients to cells within thick constructs to overcome diffusion limitations and is an essential element for realizing the potential of constructing full-scale tissues and organs [205]. With the development of complex tissue model systems, the incorporation of functional vascular networks is important as they play a key role in several biological processes including wound repair, progression of cancer and tumor pathologies, developmental angiogenesis, as well as aiding in graft implantation and integration into the host tissues [206–209]. To date, methods to produce vasculature in vitro can be divided into several strategies: (1) via the incorporation of proangiogenic growth factors (e.g., VEGF, basic fibroblast growth factor, and epidermal growth factor) to stimulate de novo vessel formation, (2) use of sacrificial materials for the construction of hollow microchannels, (3) use of microfluidic systems, or (4) patterning of encapsulated endothelial cell populations [210]. However, engineering functional blood vessels at multiple length scales within a construct ranging from small capillaries to large arteries has been a challenge in the field due to limitations in biofabrication technologies.

Recently, the flexibility and resolution offered by advanced 3D bioprinting platforms have enabled the production of vascular networks possessing complex

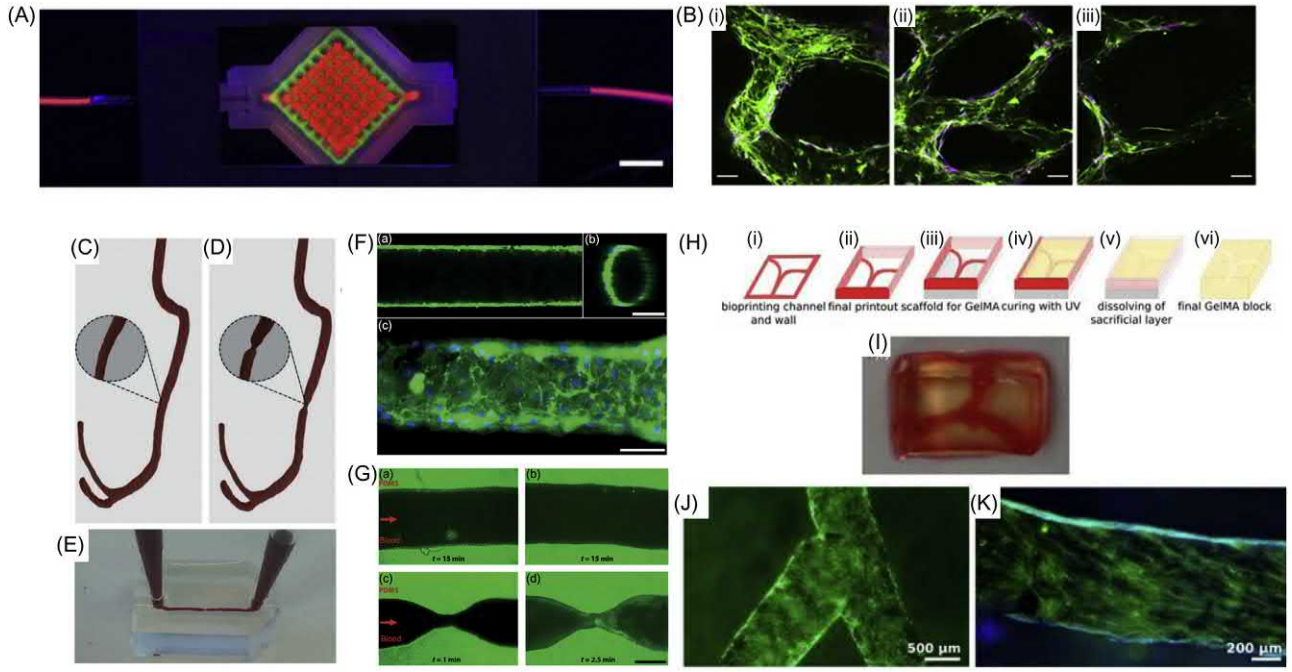

FIGURE 75.6 Vascular tissue models. (A) Image of an extrusion printed vascularized tissue contained in a perfusion chamber. Scale bar = 5 mm. (B) Fluorescent images of direct DLP-based 3D bioprinted prevascularized tissues showing stained HUVECs (*green*, CD31) and supportive mesenchymal stem cells (*purple*, alpha-smooth muscle actin) of varying widths (i–iii) ranging 50–250 μm. Scale bar = 100 μm. (C and D) Schematic of 3D printed health and stenotic vascular models, as well as (E) an image of the PDMS chip containing the vascular microchannels. (F) Confocal images of HUVECs stained with *F*-actin (*green*) and nuclei (*blue*) lining the interior microchannel walls. Scale bar = 200 μm (top and side view) and 50 μm (confluent interior). (G) Microfluidic device with healthy geometry before (top left) and after 15 minutes of blood perfusion (top right) demonstrates no evidence of platelet aggregation. A microfluidic device with stenotic geometry after 1 minute (bottom left) and 2.5 minutes (bottom right) of blood perfusion demonstrates thrombosis at the apex of the stenosis. Scale bar = 200 μm. (H) Schematic of an extrusion 3D bioprinting process to form a vascularized hydrogel thrombosis model as shown in (I). (J) Images of endothelialization within bifurcated microchannels showing (K) a confluent monolayer of CD31 (*green*) and nuclei (*blue*) stained HUVECs along the walls. *3D*, Three-dimensional; *DLP*, digital light processing; *HUVECs*, human umbilical vein endothelial cells; *PDMS*, polydimethylsiloxane. *Reproduced with permission from (A) National Academy of Sciences, ©2016 [130]; (B) Elsevier (2017) [107]; (C–F) The Royal Society of Chemistry (RSC) (2017) [218]; and (H–K) PubMed Central (PMC) (2016) [219].*

geometries over traditional approaches by way of sacrificial, spheroid-based, core–shell extrusion, and light-based printing techniques. For instance, using sacrificial biomaterials that can be evacuated to produce intricate microchannel networks and subsequently populated with endothelial cells is a simple and effective technique to create functional vessels. Using this approach, Kolesky et al. employed an extrusion-based bioprinter and formed intricate networks with a fugitive ink composed of Pluronic F-127, which was subsequently cast with a soft gelatin–fibrinogen matrix containing human neonatal dermal fibroblasts (Fig. 75.6) [130]. Following removal of the Pluronic F-127, the resulting open microchannels, approximately 400–500 μm in diameter, were seeded with HUVECs into a confluent monolayer and it was demonstrated that the perfused tissue chip was capable of supporting a tissue thickness of 1 cm for at least 6 weeks in culture [130]. In another approach, Norotte et al. utilized a scaffold-free bioprinting method that relies on the self-assembly of patterned cell spheroids to maximize direct cell–cell interactions and achieve high cellular density found in native vessels [211]. This was demonstrated by depositing spheroids of Chinese hamster ovary cells and human skin fibroblasts (HSFs) onto supporting agarose rod molds. After 5–7 days in culture to allow for cell fusion, the spheroids assembled into hollow vessels measuring 900 μm in diameter with a wall thickness of 300 μm [211]. However, issues regarding spheroid scalability, long fusion durations, and homogeneity of the final vessel upon spheroid fusion limit the use of this approach for building larger structures. As such, this group demonstrated a more efficient technique via the extrusion of multicellular cylinders composed of human umbilical vein smooth muscle cells and HSFs onto agarose rod molds, which enabled fusion to complete within 2–4 days resulting in branched tube structures with double-layered walls [211]. While promising, spatial resolution remains a challenge using this approach since it is restricted by the spheroid size or micropipette tip diameters (i.e., 300 or 500 μm). Furthermore, a direct method to construct vasculature that circumvents the multiple steps and time involved in sacrificial and spheroid-based

printing strategies would enable a more rapid, streamlined, and scalable fabrication approach. To address this challenge, Jia et al. developed a technique to directly print perfusable vascular constructs using an extrusion bioprinter fitted with a trilayered coaxial nozzle to deposit a blend bioink composed of sodium alginate, GelMA, and 4-arm PEG-tetra-acrylate (PEGTA) [212]. Ionic crosslinking of the alginate component was first induced via the simultaneous delivery of calcium ions in the core channel and ambient spray in the outer ring of the print nozzle to ensure temporary mechanical stability during the extrusion process. Afterward, the GelMA and PEGTA components were UV photopolymerized to permanently stabilize the final construct. By varying the nozzle internal and external needle sizes, different combinations of diameters (i.e., outer = 500−1500 μm, inner = 400−1000 μm) and wall thicknesses (i.e., 60−280 μm) were achieved using this technique in addition to being able to readily form various designs and geometries of perfusable networks. Furthermore, it was demonstrated that coencapsulated HUVECs and human MSCs within the blend bioink provided an optimal microenvironment for the cell viability, proliferation, and spreading to form perfusable constructs resembling native vasculature after 21 days in culture [212]. However, the ability to form multiscale vasculature structures in a continuous single step using this approach remains difficult as it will involve physically interchanging different nozzle sizes throughout the printing process. One strategy is to utilize light-based printing that does not involve physical contact during fabrication and instead works by projecting a series of digital patterns onto a photopolymerizable reservoir to quickly form vasculature structures in a direct manner. Zhu et al. demonstrated this technique by rapidly constructing complex hierarchal branched networks by using a microscale continuous optical bioprinting platform to encapsulate HUVECs and supportive C3H/10T1/2 cells within a GelMA and GM-HA blend prepolymer (Fig. 75.6) [107]. Meanwhile, the areas surrounding the printed vasculature networks were printed with a GelMA bioink embedded with HepG2 to form a heterogeneous tissue construct. Intricate vessel structures with microchannel widths ranging between 50 and 250 μm were readily fabricated and over time the endothelial cell population remodeled the hydrogel to form lumen-like structures after 1 week in culture [107]. In vivo implantation into a subcutaneous mouse model of the prevascularized tissues also revealed integration and anastomosis to the host circulation after 2 weeks compared to nonprevascularized controls [107].

With the promising application of 3D bioprinting technologies to engineer vascularized tissues, several studies have also utilized the flexibility of 3D bioprinters to create models to better understand the mechanisms of vascular pathogenesis and disorders. In particular, thrombosis has been linked as a major contributor and cause for global disability and mortality due to its association as an underlying factor for cardiovascular disorders [213,214]. For instance, venous thromboembolism, in the form of deep vein thrombosis or pulmonary embolism, is a prevalent medical condition that results in a blood clot occurring in the vein and affects 1 out of 1000 adults annually in the United States [215]. This condition derives from patient risk factors such as age, obesity, surgery, genetic factors, trauma, cancer, and immobility that can lead to chronic pain, tissue swelling, formation of venous ulcers, and in severe cases lung collapse resulting in heart failure [216]. Similarly, arterial thrombosis is also a leading cause of death in the United States due to occlusions caused by clots or arterial plaque that when left untreated, can manifest into a host of secondary health complications including stroke, heart attack, and critical limb ischemia [217]. Due to its prevalence, there is a need for models of thrombosis that accurately recapitulates the vessel geometries, local flow patterns, as well as underlying cellular interactions to develop better future treatment options. Recently, Costa et al. used a stereolithography-based 3D printer to create a replica of a miniaturized coronary artery model based on CT angiography (CTA) (Fig. 75.6) [218]. Using this approach, they were able to produce polydimethylsiloxane microfluidic chips of healthy and stenotic coronary arteries that were seeded with a monolayer of HUVECs and perfused with human whole blood containing fluorescently labeled platelets [218]. Under physiologically relevant arterial shear rates, it was demonstrated that thrombosis occurred within the stenotic geometries after 2.5 minutes of perfusion but not in the healthy controls as expected. Computational fluid dynamics further confirmed the validity of their scaled microfluidic model geometries to closely correlated to the original CTA scale geometries under the selected flow velocity profiles and shear rate distributions used in this study for both the healthy and stenotic conditions [218]. This work highlights several advancements regarding the ability to recapitulate real vessel architectures possessing smooth walls to elucidate factors affecting hemodynamics. In particular, changes in fluidic velocity gradients, increased shear rates, platelet formation in the recirculation zone, and activation of the intrinsic coagulation pathway within stenotic vessel geometries were probable root causes of thrombosis in their model [218]. In another study of thrombosis, Zhang et al. examined the pathology of fibrosis within vessels by using an extrusion-based 3D printer to create hollow bifurcated microchannels within a GelMA hydrogel via the removal of sacrificial Pluronic F-127 (Fig. 75.6) [219]. To evaluate the migration of fibroblasts in exacerbating the formation of a fibrotic clot due to a damaged endothelium three groups were tested:

(1) microchannels lined with HUVECs and no fibroblasts in the GelMA matrix as a negative control, (2) HUVECs lined microchannels with fibroblasts in the GelMA matrix to serve as a normal vessel control, and (3) fibroblasts in the GelMA matrix with no HUVECs lining the microchannels to simulate the damaged endothelium [219]. Thrombosis was induced by administering $CaCl_2$ into human whole blood which was then perfused into the microchannels for each group and allowed to culture for 2 weeks. Interestingly, as expected there was no visible staining of collagen I present within the clot in both the negative control and normal vessel control due to the absence of fibroblasts and barrier formed by the endothelium, respectively. In contrast, the group simulating the damaged endothelium had significant invasion of fibroblasts found in the regions of the thrombus along with deposition of collagen I secreted by the cells, which is a representative of the in vivo setting [219]. Overall, these realistic models hold great potential to serve as physiologically relevant platforms for studying the mechanisms of thrombus formation as well as other vascular disorders by being able to closely mimic the native vascular microenvironment.

Conclusion and future directions

Over the years, 3D bioprinting has surfaced as a flexible biofabrication tool that enables the creation of complex tissue model systems to better recapitulate the native microenvironment. In particular, the successful outcome of engineered tissue models relies on several key factors including mechanical properties (i.e., stiff/soft and elastic/brittle), biochemical composition (ECM components, growth factors, etc.), and complexity of the final tissue (cell source, multiple cell types, shape, and organization). Taken together, careful consideration of these factors can influence which method of 3D bioprinting is best suited for a specific tissue application. To date, a growing variety of engineered tissues have been successfully produced using a range of different 3D bioprinting modalities to serve as models for improving diagnostic and therapeutic outcomes including nerve, cancer, heart, liver, and vascularized tissues as highlighted above. While these advanced tissue models represent a step closer to physiologically relevant tissue platforms, many challenges remain regarding their maturation state, multicellular complexity, and scalability for high throughput manufacturing.

With the emergence of complex 3D tissue models, the trend toward the selection of stem cell–derived cells, particularly iPSCs, has gained popularity in terms of their practicality in research and translational advantages. While promising, it is important to recognize that the relevancy and performance of tissue models composed of these cell types are highly dependent on the efficacy of the protocols employed to control differentiation and maturation toward the desired lineage in vitro. Specialized cell types differentiated from iPSCs are not equivalent in their maturation state as primary cells and are considered immature with resemblance more similar to fetal cells of the native tissue. As such, the successful use of stem cell–derived cells hinges on new methods to facilitate their differentiation pathway to achieve mature adult phenotypes and functionalities in order to more accurately extrapolate data for disease processes observed in adult tissues and organs. In another aspect the multicellular population inherent in native tissues comprised of both parenchymal and nonparenchymal cell types is critical in the design of physiologically relevant tissue models. Simplistic 3D tissue models contain a homogenous population comprised of a single cell type representing the functional unit of the tissue or organ. However, growing evidence in recent literature has shown that supportive nonparenchymal cells play a critical role in overall tissue function [108,203]. As such, future designs will need to continue to recapitulate tissue or organ cellular heterogeneity to build toward improved engineered tissue models that possess greater relevancy to native behavior that will increase their efficacy. Finally, reliable and reproducible manufacturing methods will be an essential factor for the scalability of 3D bioprinted tissue models in high throughput applications. This is necessary as future tissue models will need to be produced in a manner that is able to interface directly with existing commercial platforms accustomed in industry, such as for drug screening and diagnostics, without compromising performance, consistency, and predictive value. Considering these factors is an important step toward achieving the full potential of 3D tissue models for use in the development of new therapies and elucidating pathological mechanisms. Nonetheless, technological advancements in 3D bioprinting continue to play a critical role in defining new directions and possibilities for realizing the production of tissue or organ substitutes in both research and clinical applications.

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Chapter 76

Body-on-a-chip: three-dimensional engineered tissue models*

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Introduction

Until very recently, experimental models for human physiology have been limited to animal models and two-dimensional (2D) cell culture systems that have not proven to be reliable for accurately modeling human pathology or predicting drug efficacy or safety. There is a considerable need for a much more accurate in vitro representation of human biology for disease modeling, drug development, and determination of drug safety [1,2]. Preclinical animal models have represented the cornerstone of biomedical research for centuries. These animal models have been instrumental in a plethora of discoveries across virtually all areas of science [3]. However, it has become clear that animal models have significant limitations due to phenotypic differences in physiology as compared to humans. This is especially critical for the assessment of toxic side effects of drugs that might target the liver, and other organs, due to differences in enzyme isotype expression profiles between humans and animals; often resulting in significant differences in drug metabolism, efficacy, and toxicity.

2D cultures of transformed or primary cells have also been used extensively as models of human biology. These in vitro models do normally use human cells; however, the genotype of cell lines is, by definition, altered from the natural state. Primary cells isolated from cadaveric tissues can undergo dramatic phenotype changes when cultured on a rigid plastic substrate. These changes occur as cells attempt to adapt to a radically new environment that lacks many of the physical and biochemical features of native tissue [4,5]. Some of the most prominent features of the normal cellular microenvironment lacking in static 2D culture are cell/cell and cell/matrix interactions, the stiffness of the cell substrate, and the lack of an interstitium to facilitate appropriate autocrine and paracrine signaling [4,6,7]. The latter of these also results in the inability to accurately model drug diffusion kinetics resulting in erroneous estimations of threshold doses for efficacy or toxicity [6,7].

Recently, more advanced in vitro models have been developed that incorporate many of the basic principles of tissue engineering. These include creating a three-dimensional (3D) cell structure containing multiple cell types within a supportive biomaterial scaffold that has appropriate mechanical properties. In combination with advanced microfluidic and microelectronic technologies, these in vitro models demonstrate many of the functional properties of normal human tissue and organs. For example, liver models exhibit normal metabolic activity, skeletal and cardiac muscle constructs contract in a physiologically normal manner, lung organoids "breath," and gut/vessel/brain microvasculature constructs maintain normal barrier functionality [8,9].

"Body-on-a-chip" devices that recapitulate 3D tissue architectures and physiological fluid flow conditions are much more supportive of normal cell function than static 2D culture [10]. These engineered platforms can include sophisticated hardware systems with the potential for scaled-up automated production that increase the potential for high-throughput experimentation and screening of test compounds. These models deliver much more sophisticated cellular interactions and responses while providing user control over physical factors such as fluid shear stress and mechanical stresses. Microfabrication techniques based on a variety of technologies have resulted in

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the development of microscale fluidic systems with predictable fluid dynamics throughout the entire fluid circuit [11]. Tissues and organoids can be immobilized at specific locations within platform microreactors using advanced hydrogel biomaterials. These materials provide a proper microenvironment and anchor the cellular constructs within the fluidic circuit for long-term culture. A variety of iterations upon these basic concepts are currently being utilized by laboratories around the world [12–14]. In addition to modeling normal human physiology, a variety of on-chip disease models have also been investigated [13].

The current and most pressing challenge in advancing the body-on-a-chip field is the integration of multiple tissue types within a common microfluidic circuit. Such systems represent a major step toward in vitro modeling of human physiology at the organism level. Ideally, these systems would recapitulate the interdependent and interrelated functions of all tissues and organs within the human body. The microfluidic circuit connecting organoid microreactor chambers allows for fluid flow across each organoid type in a sequence in much the same way that the vasculature delivers blood sequentially to the organs within the body [8]. Drugs, metabolites, and soluble molecular factors would be transported to downstream tissue model types in a physiologically relevant sequence. These advanced body-on-a-chip platforms would be ideal for testing newly developed drugs and determining potential toxic side effects in humans. Furthermore, integrated body-on-a-chip platforms would offer tremendous benefits for pharmacological studies aimed at determining the specific effects and toxic thresholds for newly developed drugs, allowing for better prediction of appropriate doses for human trials. In this chapter, we highlight a variety of body-on-a-chip systems for applications such as drug screening and disease modeling and look to the future of multiorganoid body-on-a-chip systems and applications in personalized precision medicine.

Advanced in vitro modeling systems—progression from two-dimensional to three-dimensional models

The development of novel drugs that are safe and effective in humans has been slowed by the lack of accurate models for human physiology. Animal models, used extensively in preclinical drug studies, are traditionally regarded as the gold standard for establishing drug safety. However, animal models do not accurately reflect human drug metabolism and often yield results not predictive of results in humans. As discussed earlier, conventional in vitro 2D model systems lack many of the environmental questions required for the long-term maintenance of cell viability and cellular function [4,15]. In addition, drug distribution and cellular drug bioavailability are completely nonphysiological in traditional cell culture, and drug doses that are effective in 2D are often ineffective when scaled to patients [6,7].

Recently, an appreciation has developed for applying basic principles of tissue engineering to in vitro models. A myriad of studies have demonstrated that 3D cell culture consistently outperforms 2D cultures in many aspects, including maintenance of in vivo function, including metabolism of drugs and toxins [16]. The current drug development pipeline (Fig. 76.1A) does not yet include advanced 3D models for human physiology, and countless discrepancies between in vitro drug screening and clinical performance continue to be identified [17]. As an example, it has recently been demonstrated that metastatic colon carcinoma cells adopted an epithelial appearance in 2D tissue culture. However, when transitioned to a 3D form factor, the cancer cells adopted a morphology that more closely resembled malignant cells, in vivo [18]. Observations such as these raise the question, why is there not greater momentum to include advanced 3D models into the clinical translational pipeline?

Tissue engineering technologies have evolved to the point that microengineered tissue constructs can better mimic the architecture, cellular diversity, and function of in vivo tissue. In vitro models incorporating principles of tissue engineering can often be maintained in viable states and preserve physiologically relevant levels of function for periods of time that greatly exceed those possible in 2D. It is generally assumed that tissue engineering technologies, including construct geometry, cellular scaffolds/substrates, mechanical properties of the microenvironment, and cellular diversity, all contribute to the increased function of in vitro physiological models. Oxygenation is seen as a limiting factor in the construction of 3D tissue models. When these constructs become larger, an oxygen gradient develops across the cellular model, which can lead to heterogenous phenotypic changes across the construct. However, oxygen gradients exist within organs and tissues, in vivo. As such, so long these gradients and their effects are characterized, they may actually increase the reliability of the model. When 3D models become too large, insufficient oxygen diffusion to the core of the construct can result in cellular necrosis. This may only be prevented by limiting the size of the constructs, or by incorporating a channel system, through which the cellular model may be perfused.

An area in which the use of 3D cell culture systems has seen significant growth is in basic research. Academic science in particular is beginning to embrace 3D models. However, 2D cell culture has been an established technology for many decades and will certainly remain a widely used tool for many years to come. 2D cell culture is easy and inexpensive in comparison to 3D culture systems. Implementing 3D systems in a lab can be complicated

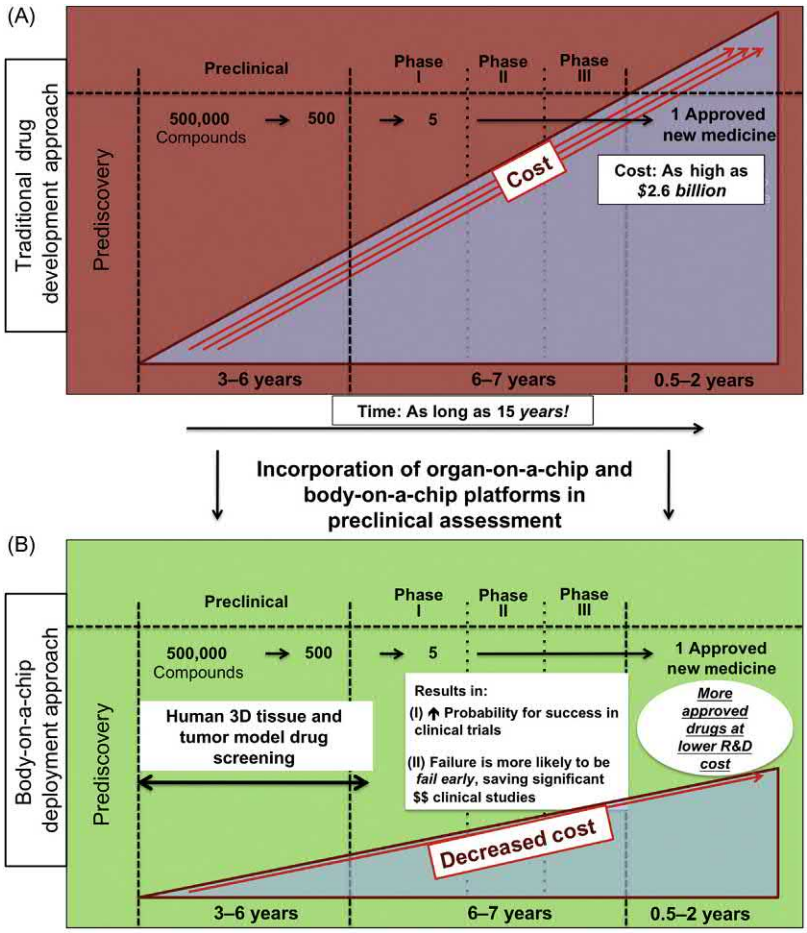

FIGURE 76.1 Potential improvements in the drug development pipeline as a result of deployment of organ-on-a-chip and body-on-a-chip technologies into pharmaceutical research and development. (A) The current drug development pipeline requires many years and multiple billions of dollars to bring a drug to market. (B) Plugging in human-based biofabricated on-a-chip platforms into preclinical stages can potentially drastically improve the efficiency of the drug development pipeline.

and require training in several new technologies, including biomaterial development and biofabrication techniques. After the initial learning curve has been overcome, several challenges persist. Processes regarded as trivial in 2D culture, such as cell harvest and cell passage, can be quite difficult and in some cases impossible in current 3D culture systems. For example, if cells are cultured within a 3D hydrogel, the substrate must be dissolved in order to harvest the cells. Some biomaterials support cell retrieval by building in features that allow for easy liquification of the substrate for cell harvest [19]. Most semisolid substrates still require enzymatic dissolution that can be quite damaging to the cells, and result in significant reduction in viability. In addition, a majority of the cell imaging techniques were developed for 2D cell cultures. These techniques were designed to image cells across a narrow focal plane. In 3D, cells also must be imaged across the *Z*-axis. Consequently, high-quality imaging in 3D may only be obtained by fluorescence imaging that may be activated within a specific plane of space. These techniques, including confocal or macro-confocal microscopy, require expensive equipment that many laboratories do not have access to. In addition, several assays routinely used to characterize cells in culture are significantly more difficult to perform on 3D models or require significant adaptation for application to 3D models. Finally, some materials used in body-on-chip systems, particularly polydimethylsiloxane, are prone to fouling and drug and protein adsorption. However, new materials for device hardware are being developed to solve this problem.

After considering all things, 3D systems represent a better strategy for modeling normal human physiology, as compared to 2D cell culture [16]. These 3D platforms have an immense potential to improve several aspects of biomedicine. In particular, 3D models may accelerate drug candidate evaluation and reduce the number of toxic compounds that make their way deep into the translational pipeline—decreasing development costs and increasing success rates of clinical trials (Fig. 76.1B).

Organ-on-a-chip technologies and their applications

In recent years, advances in tissue engineering [20], biomaterials [21], and micro-biofabrication [22] have allowed for the development of fabricated tissue analogs that reflect native tissue function to a degree that was not possible just a few years ago. A wide variety of advanced

human in vitro models have been developed and validated for modeling the effects of drugs and toxins [14,23–25]. Advances in molecular genetics and tissue engineering technologies have also allowed for the development of 3D models of human diseases [13,26–28]. Technologies adjacent to tissue engineering, such as microfabrication, and microfluidics, have augmented 3D cell models by providing precisely controllable fluid flow, in-line biosensing and platform miniaturization for high-throughput testing. These organ-on-a-chip systems vary widely in design and cover a wide range of tissue types. Some of these systems have already proven beneficial in drug discovery [12] and are beginning to show promise for improving the efficiency of the clinical translation pipeline. The following represent just a few examples of microengineered tissue models, including, liver, vasculature, lung, and tumor. However, it should be noted that there are many variations of these systems, as well as many additional models for virtually any tissue type.

Microengineering and biofabrication

The cellular component of a body-on-a-chip model may be considered the fundamental core of the construct. However, including a physiological composition of cell types alone does not guarantee recapitulation of normal cellular function. An additional component of the cellular microenvironment that must be considered is the extracellular matrix (ECM). As described previously, the cellular microenvironment has a direct effect on cell phenotype. Therefore it is important to include as many components of the normal cellular microenvironment in an in vitro model system as possible. Several strategies have been developed to include both the structural and biochemical aspects of the cellular microenvironment in advanced 3D model systems.

The technique of micropatterning refers to the precise deposition of bioregulatory molecules within a cell culture substrate. Micropatterning can be accomplished by a variety of methods including (1) microcontact printing, wherein a stamp, coated with a pattern of ECM proteins, is pressed against a solid substrate; (2) photo-patterning using ultraviolet light projected through a photomask to catalyze adherence of ECM proteins in a predetermined pattern; and (3) laser-patterning, where laser light is used to mediate protein binding to a substrate in any desired pattern with very high resolution [29].

Many bioregulatory components of the ECM can be distributed in a controlled manner by using micropatterning techniques. For instance, deposition of discrete units of ECM cell proteins that restrict cell spreading induces apoptosis in bovine adrenal capillary cells, while simultaneously maintaining the differentiation state of epidermal keratinocytes. Conversely, micropatterning of ECM proteins that induce cell spreading promoted the proliferation of both the bovine adrenal capillary cells and epidermal keratinocytes. These experiments demonstrate a clear link between the local ECM composition of a cell substrate, cell cycle, and cell differentiation potential [30,31]. This concept is further supported by another study where mesenchymal stem cells grown on small micropatterned patches that restrict cell spreading promoted adipogenic differentiation, whereas micropatterning of factors that induce cell spreading promoted osteogenic differentiation. These studies also showed that modulation of cell shape was sufficient to induce the expression of the signaling proteins Rac1 and *N*-cadherin, which govern cell lineage specification [32,33]. Micropatterning represents a powerful tool for precisely controlling protein composition of the cellular microenvironment within a cell culture substrate. By patterning the biochemical properties of a cell substrate, the phenotype of cells within that substrate may also be patterned. This opens the potential for controlling cellular microarchitecture within the construct. Micropatterning has a wide variety of applications in advanced in vitro modeling and will become increasingly utilized for fine-tuning the cellular arrangement within these systems.

The micropatterning technologies described earlier are most appropriately used to control the cellular microenvironment in a 2D plane. Bioprinting provides a method for doing the same in a 3D space. Bioprinting involves the layer-by-layer deposition of structural material, cells, and bioregulatory factors in a controlled manner. Current bioprinting technologies allow for the fabrication of complex cellularized 3D constructs that may include components of the ECM, including both intrinsic and bound bioregulatory.

Bioprinting technologies are highly customizable across a wide range of both resolution and physical characteristics. Applications requiring a more rigid structure can be printed using biomaterials with high mechanical stiffness. For stiffnesses beyond those that are compatible with a specific printing modality, methods for cross-linking structural components of the biomaterial subsequent to bioprinting have been developed. As an example, very rigid dental implants have been bioprinted from polycaprolactone and hydroxyapatite [34]. On the other end of the stiffness spectrum, soft tissues, such as vascular grafts, have been printed using very low stiffness poly(ethylene glycol) hydrogels [35]. With increasing resolution and speed of modern bioprinters, the structures that may be fabricated are becoming highly complex. The ability to precisely pattern the cellular, structural, and biochemical microarchitectures of tissues will most certainly increase the fidelity and function of bioprinted 3D cell models.

Several 3D cell models have already been fabricated using bioprinting technology. Liver constructs that

include multiple liver cell types within a supportive hydrogel have been generated using microextrusion technology. These constructs demonstrated exceptionally high functionality and viability over the long term [36]. Skin analogs have also been created using a laser bioprinting technology. This technique allowed for layered deposition of different cell types within the engineered skin. The resulting constructs were implanted into rodent dermal wounds and demonstrated robust neovascularization, differentiation of mature keratinocytes, and generation of a normal dermal basal lamina—all hallmarks of native skin [37].

3D printing may still be considered an emerging field. Logistical obstacles continue to limit the potential for whole organ biofabrication. However, the speed, reproducibility, and scalability of bioprinting make it an ideal fabrication technology for body-on-a-chip modeling. 3D printing can be used to generate an industrial-scale volume of constructs with low run-to-run variability and the complex architecture required for high fidelity modeling.

Liver-on-a-chip

Early tissue-on-a-chip devices were designed to promote cell aggregation, thereby creating 3D cell structures. For example, microwells with a convergent geometry were designed to funnel cells together into a compact structure. Based on the microwell geometry, liver cells could be formed into either spherical or cylindrical constructs. These 3D constructs maintained much better cellular function than 2D controls [38,39]. In another example, spheroids were created within an array of channels connecting inverted pyramid-shaped microwells, allowing for the delivery of cells and test compound to multiple chambers, simultaneously. This integration of microfluidics with an array of microwells greatly increased the throughput potential for experimentation using 3D constructs [40].

In recent years, liver-on-a-chip devices have become much more complex. Currently, they may employee controlled fluid flow for addressing nutrient circulation, drug administration, sample collection, and integration with other tissue types. The latter will be discussed in detail later in this chapter. In one such liver-on-a-chip, hydrogels were used to encapsulate HepG2 hepatoma cells with NIH-3T3 fibroblasts. These arrays of 3D organoids demonstrated increased liver function, as compared to 2D controls, and produced an appropriate toxic response to acetaminophen [41]. In other experiments a versatile photopolymerizable hyaluronic acid biopolymer system was used for in situ photo-patterning of HepG2 cells to generate 3D liver constructs. The constructs were formed in parallel channel fluidic devices that were fabricated by soft lithography and molded PDMS. This system was used for toxicity screening by administering multiple alcohol concentrations within each chip. The expected dose response was seen for both viability and cellular function [14]. Current efforts within our group are focused on the miniaturization of these types of systems, to further increase throughput. Miniaturization and microfabrication approaches can be employed to generate more intricate biological microarchitecture, perhaps at a high enough resolution to model capillary structures. Precise layering of hepatocytes and endothelial cells within microfluidic circuits may be used to generate structures with the resolution required for modeling sinusoid-like structures [42]. Another approach for generating biologically relevant microarchitecture involves mating synthetic and biological components. As an example, using a semiporous membrane to separate two adjacent chambers may be used to partition human hepatocytes from sinusoidal endothelial cells. Such a design was shown to generate higher levels of albumin and urea, as compared to traditional hepatocyte cultures, and demonstrated another strategy for recapitulating normal microarchitecture to increase cell function [43].

Vessel-on-a-chip

The term “microfluidics” carries with it the assumption of controlled fluid routing. Thus microfluidic devices are effective for modeling vascular networks. Drugs are generally either introduced directly into the blood stream or enter the blood stream shortly after oral or pulmonary intake. Microfluidics are used to model the trafficking of compounds and metabolites within a body on a chip platform. A substantial number of vascular fluidic devices have been developed, including both straight channel devices [44,45] and devices with more complex, branching geometries [46,47]. A major feature of the vasculature is to form a barrier between the circulation and the tissue interstitium at the capillary bed. Many microfluidic systems have been designed to model transendothelial delivery of test compounds to a target tissue. As an example, two perpendicular channels that cross at a single point were constructed with a semipermeable membrane colonized by endothelial cells positioned at the point where the two channels cross. Fluorescently labeled albumin was introduced into one channel and transport through the endothelial monolayer that was quantified by laser excitation of the fluorophore in the other channel [48]. In another example an endothelialized construct was designed with a mechanism that allowed for the control of shear stress experienced by the endothelial cells. The device was used to determine the effect of fluid shear on nanoparticle translocation across the endothelial monolayer. These studies were intended to define the ability of fluid shear to influence pharmacokinetics and drug

biodistribution [49]. Other microfluidic devices with integrated vasculatures have been developed to determine how the atomic structure of drugs and nanoparticles can influence the rate of translocation across the capillary bed [50]. The integration of vascular function in organ-on-a-chip microfluidic design would seem to offer great promise in providing more accurate modeling of drug pharmacology in next generation in vitro cell platforms.

Lung-on-a-chip

The lungs, which represent a fluid/air interface between the in vivo and ex vivo environments, serve as a port of entry for drugs, toxins, pathogens, and other xenobiotic compounds. Accurate modeling of the alveoli in organ-on-a-chip systems is an important component of modeling the effects of compounds that enter the circulation through the lung. Significant advancements in the on-chip modeling of lung tissue have been realized over the past decade [51]. Many of these lung constructs consist of lung epithelial cells and endothelial cells situated on opposing surfaces of a semipermeable membrane. The cellularized membrane forms a barrier that can model transport of aerosols or vapors from the gaseous alveolar compartment into the aqueous circulatory compartment. Contact of the alveolar epithelial with air in the alveolar compartment has the added advantage of promoting normal cellular function and maintenance of the mature differentiation state. In more complex models, multiple independent pneumatic channels were incorporated into the design. Cyclic deformation of the pneumatic channel walls paired with controlled shear within the fluid channel promoted exceptional cell morphology and function [52,53]. These advanced models have proven to be quite valuable for modeling several lung pathologies, including inflammation, pulmonary edema, mucus plug rupture, alveolar epithelial cell damage, and have also improved the reliability of drug screening [54–57]. While planar air/fluid interface models have shown incredible promise for modeling normal exchange across the alveoli and certain pulmonary disease states, it is worth noting that more simple, acinar lung organoids may be sufficient for screening drug toxicity of compounds delivered orally or directly into the circulation.

Heart-on-a-chip

Current models for cardiac tissue are generally quite straightforward in design. The heart's sole function in the human body is to drive the circulation of blood, and a majority of the in vitro cardiac models are designed around modeling this function. Simple monolayer cultures of human cardiomyocytes will spontaneously beat in culture when grown on Matrigel [58]. Sheets of human cardiomyocytes may be layered to produce 3D cardiac constructs that retain the ability to contract in synchrony [59]. These planar construct designs are sufficient for modeling the heart's beating action but are not ideal for modeling 3D mechanics such as contractile force. 3D cardiac constructs consisting of human cardiomyocytes embedded in ring-shaped collagen hydrogel structures have been shown to self-organize into a circumferentially aligned architecture that supports physiologically relevant action potential propagation [60]. These types of constructs have been integrated into microfluidic circuits to form dynamic, contractile heart-on-a-chip systems [61–65].

Cancer-on-a-chip

In addition to modeling normal tissue such as liver and heart, excellent models for tumor tissue have also been developed. These models have been integrated into microfluidic platforms to form tumor-on-a-chip devices capable of modeling both tumor growth and metastasis. The microenvironments of tumors are often very complex and may vary significantly from tumor to tumor. In addition, the malignant properties of the cells within the tumor are often reliant upon the nature of the stroma. The physical and biochemical characteristics of 3D tumor models can be controlled using microfluidic and microfabrication techniques. Tumor models derived from a patient's tumor sample can be used for determining effective chemotherapeutic agent selection and determining an optimal dose on a patient-by-patient basis [66,67].

Recent advancements in tumor-on-a-chip modeling include the development of integrated hardware for monitoring the tumor tissue. These include advanced imaging technologies and onboard molecular assays, which provide detailed characterization of tumor behavior, on-chip. The microscale of these types of models has been shown to significantly influence cell metabolism. This results from the bioavailability of oxygen within these platforms. Studies have demonstrated that microfluidic systems provided greater access to oxygen, as compared to standard 2D culture systems. This increased oxygen level results in increased Krebs cycle activity and decreased expression of hypoxia-regulated factor-1 [68]. Constant perfusion of with oxygenated media provides a better model for a normal tumor microenvironment.

A device has also been designed with multiple drug gradient generators and parallel cell culture chambers to facilitate multidose high-throughput drug screens. This system has been paired with automated cell labeling and high content imaging, on-chip [69]. Another tumor model design includes microscale bioreactors that contain hepatocytes, nonparenchymal cells, and breast cancer cells and is intended to simulate the liver microenvironment.

The device also contains oxygen sensors, micropumps for controlling nutrient distribution, and real-time sampling capabilities [70]. This device was used to demonstrate that breast tumor cells will spontaneously become dormant when placed within the microenvironment of the liver. This effect was suspected to result from the cytokine profile created by the presence of hepatic cells within the liver portion of the construct. Breast cancer has also been studied using a system that includes models for both the ductal and lobular components of breast tissue [71,72]. This system is intended to model the interaction between these two microanotomical compartments during tumor initiation and progression. In another device, HCT-116 colon carcinoma cells and HepG2 hepatoma cells were encapsulated in Matrigel in separate chambers. Myeloblasts were embedded in alginate gels within an additional chamber in order to simulate bone marrow. Using this platform, the cytotoxic effects of the 5-fluorouracil (5-FU) prodrug Tegafur could be determined for each cell type. Interestingly, using 3D tumor organoids, the liver constructs were able to metabolize Tegafur to 5-FU, resulting in cell death within the other two tumor organoid types. Tumor models arranged in 2D were unable to metabolize the prodrug to its activated form [73].

Lung tumor models have also been developed in microfluidic devices. Human nonsmall cell lung cancer in both 2D and 3D cell model configurations were evaluated for sensitivity to several common chemotherapeutic agents [74]. In another example, lung cancer spherical constructs were formed from cell lines or derived from patient lung tumor biopsies, both with and without the addition of the associated pericyte population. Each of these constructs was tested for susceptibility to the drug cisplatin. Systems that included the pericyte population demonstrated higher levels of chemoresistance to the anticancer drug, indicating the importance of, including all relevant cell types from within the tumor when developing a tumor model [75]. These examples of tumor-on-a-chip models demonstrate the benefits of 3D design and advanced microfluidic technologies for cancer research and selection of an effective drug and dose for a specific patient.

Body-on-a-chip: integrated multiorgan systems and future applications

While body-on-a-chip technologies are relatively new, they have already demonstrated exceptional promise for applications in research and drug development. Recently, systems of increased biological complexity have been developed that feature multiple engineered tissue types integrated within a single platform [76–79]. These multitissue models [80] have demonstrated greatly increased potential for modeling physiology at the organism level, as compared to single tissue models. Until recently, these integrated platforms utilized cell lines and animal cells [73,81] More recent systems have begun to use human primary cells. These models have required the development of more advanced cell substrates and microfluidic devices to support the viability and function of human primary cells. There have been several notable studies demonstrating complex human multitissue systems. In one such system a four-tissue circuit was developed in a pumpless microfluidic perfusion platform, housing 2D cultured liver, cardiac, skeletal muscle, and neuron, all integrated within a single microfluidic circuit. This platform was designed as a screening tool for determining cell toxicity in experiments using doxorubicin, atorvastatin, valproic acid, acetaminophen, and *N*-acetyl-*m*-aminophenol [82]. The pumpless concept has also been employed in a dual-tissue gut/liver system, including transepithelial electrical resistance sensors, for monitoring gut epithelial barrier function [83]. In another study a multitissue microfluidic model was developed with integrated intestine, skin, liver, and kidney tissues. In this system function, appropriate gene expression, and viability were maintained for 28 days [77]. These examples represent important steps towards modeling the complex, multitissue responses and interactions that occur within a living organism.

The importance of multiorganoid integration

In vitro models that accurately recapitulate human tissue function are still quite rare. Even fewer are designed to model multiple tissues within a single integrated platform. Such models offer clear benefits in determining drug toxicity, as toxic responses often involve multiple tissue types. In addition, integrated multitissue models have the potential to increase function of the individual tissues to an even higher level, as endocrine and other molecular signals produced by various organs would condition the media with a more normal repertoire of supportive factors. With respect to drugs, effects in off-target tissues can be as important as effects at the intended target site. If undetected, detrimental secondary effects can lead to failure of expensive clinical trials, or withdrawal from commercial or clinical.

Multiorganoid platforms are also useful for disease modeling. Cancer metastasis, in which malignant tumor cells migrate to a secondary site, may be modeled in multiorganoid platforms. While useful for many applications, single-organoid models have limited ability to model these types of physiologically significant events. Here we describe several examples of multiorganoid platforms that demonstrate the importance of these systems.

Cancer

As described earlier, cancer metastasis is a disease process that may only be modeled in a multiorganoid system. When a tumor becomes metastatic, certain cells within the tumor gain the ability to penetrate the endothelium and enter the circulatory or lymphatic systems. They may then migrate to a distant tissue and reenter tissue at a distant secondary site. Few in vitro systems have been developed that are able to accurately model this process. One system has demonstrated that it is possible to model the metastatic process, in vitro. This metastasis-on-a-chip platform was designed to allow tracking of the migrating metastatic tumor cells from a bioengineered colon model to a bioengineered liver model within a simple recirculating microfluidic device (Fig. 76.2A). It was shown that metastatic colorectal cancer cells were able to migrate out of the colon tumor model into the microfluidic circuit and engraft in the downstream liver model. Conversely, a nonmetastatic colorectal cancer cell type continued to grow at the primary site but never migrated to the liver [84].

Tumor metastasis-on-a-chip platforms, as previously described, may comprise multiple organoids that allow for tumor cells to metastasizes from a primary location to a secondary site. On-a-chip devices have also been designed to assess certain discrete aspects of metastasis. For example, a recently developed system includes a microfluidic device that can model the process by which multicellular tumor aggregates migrate through both a collagen matrix and an endothelial cell layer [85]. Another device includes and endothelial cell layer that partitions a microfluidic circuit from a chamber that houses a 3D bone construct. This system allows modeling of the extravasation of metastatic tumor cells from the vasculature into bone [86,87]. Other recently developed models include a system for assessing the effects of interstitial pressure on cell migration [88], and a system for screening antiangiogenic drugs [89]. These systems illustrate the potential benefits that on-a-chip cancer technologies are capable of delivering. However, few platforms model both the primary and metastatic sites, as well as the barrier structures that separate these locations, such as basement membranes, blood, ECM, and endothelium. By including circulating flow through a system containing multiple organoids, modeling the migration of cells from the primary tumor into a downstream target tissue may be accomplished. The results of these systems have been very similar to what is seen clinically. For example, these systems have demonstrated colorectal cancer cells preferentially engrafting into liver constructs, a well-known target tissue for colorectal metastases [84]. These examples represent several components of the metastatic process that have been modeled in multiorganoid systems, and future studies will likely rely upon these types of platforms to uncover other factors that influence metastasis.

Drug testing/toxicology

As has been discussed throughout this chapter, a major area of interest within the body-on-a-chip field is the determination of drug safety. Multitissue type models offer particular benefits for these types of studies. For example, 5-FU is one of many common chemotherapy agents employed in treating colorectal cancer.

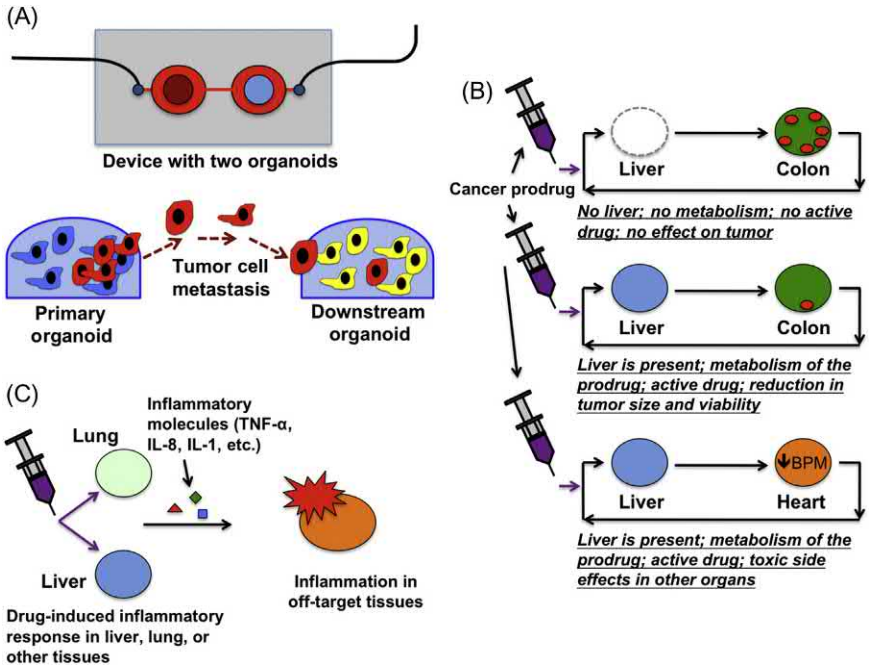

FIGURE 76.2 Examples of multiorgan interactions that cannot be modeled with single-organoid systems. (A) Migration and metastasis of tumor cells from one organ or organoid site to another, demonstrated in vitro in a metastasis-on-a-chip device in which colorectal carcinoma metastasizes from the colon to the liver. (B) Reliance of a prodrug therapy such as the anticancer 5-fluorouracil prodrug Tegafur on liver metabolism to activate the drug to generate a positive effect by successfully targeting tumor cells. (C) Inflammatory molecules secreted from organs such as the liver and lung upon drug injury can cause detrimental inflammatory responses and cell injury in downstream tissues.

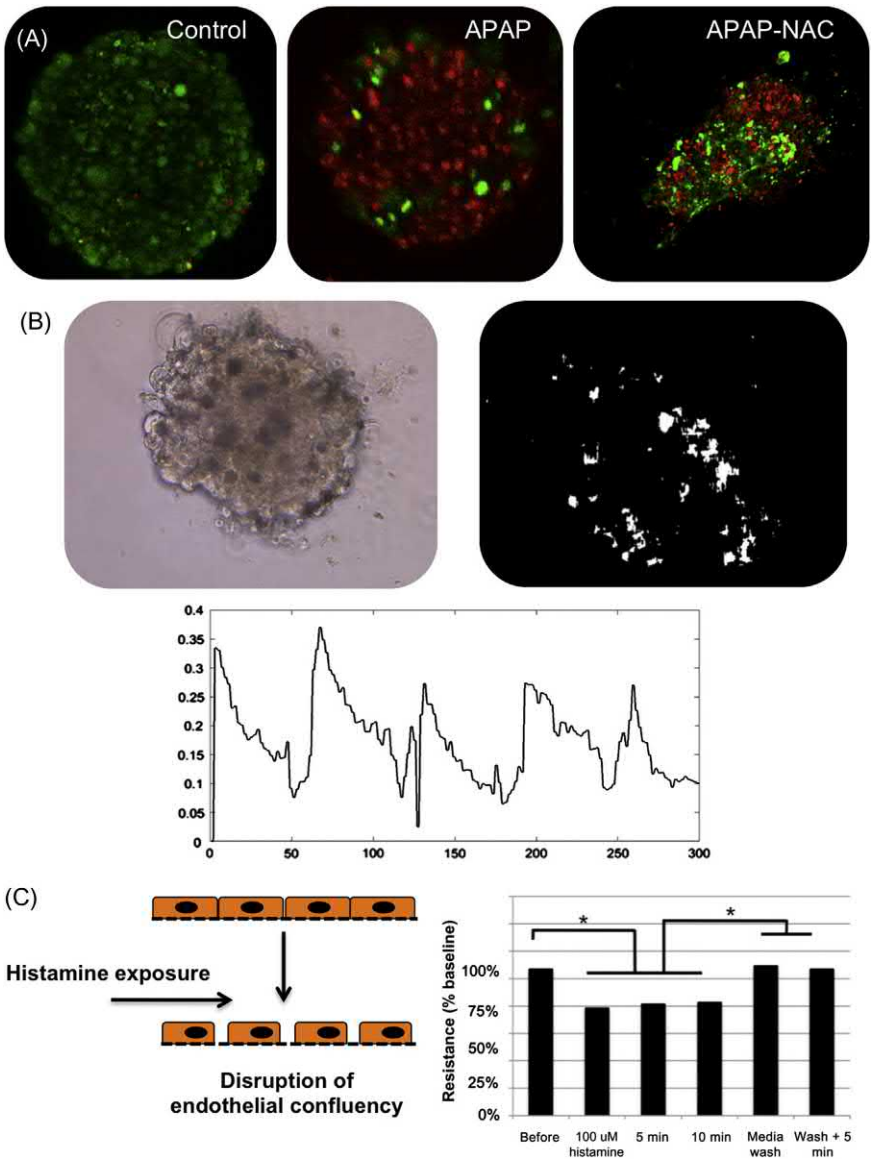

FIGURE 76.3 Highly functional organoids for a multiorganoid body-on-a-chip platform. (A) acetaminophen (APAP) toxicity in liver organoids and reduction in toxicity by *N*-acetyl-L-cysteine (NAC). (B) Cardiac organoids remain viable long-term and support transport of fluorescent dyes [lucifer yellow (*yellow stain*) and fluorescein (*green stain*)] through interconnected ion channels suggesting high levels of cell−cell communication. (C) Beating analysis of cardiac organoids: an onboard camera captures video of beating organoids, after which beating rates are calculated by quantifying pixel movement, generating beat plots. (D) Vascular endothelium devices respond to changes in endothelium integrity as measured by a transendothelium electrical resistance sensor. *NAC*, *N*-Acetyl-L-cysteine.

Unfortunately, 5-FU can induce a variety of detrimental side effects in patients, including cell damage in the gastrointestinal tract. To reduce toxicity, several prodrugs have been developed, such as Tegafur. Tegafur and other prodrugs are inactive in the administered form. The prodrug is metabolically activated by the liver to the active drug, 5-FU. Consequently, without including a functional liver model in the system, no active drug would be produced and experimental results would be irrelevant. By including a functional liver construct, along with intended target tumor cells and potential off-target tissues that may experience toxicity, a more complete understanding of the benefits and risks associated with administration of these types of drugs may be determined (Fig. 76.2B).

In another example, 3D cardiac and liver constructs were used to screen drugs and toxins. By employing a mini-microscope with custom-written software to analyze cardiac beating kinetics, precise determination of beat frequency and amplitude can be recorded [64]. These systems were used to screen a panel of environmental toxins and a set of drugs that were recalled from the market due to unanticipated toxicity in human patients (Fig. 76.3). Fig. 76.4 highlights some of these studies, where the dual-organoid systems were able to model these toxicities. In addition, the integration of different microengineered tissue types into a single system allows for screening studies that can identify unanticipated toxicities (Fig. 76.2C) [90].

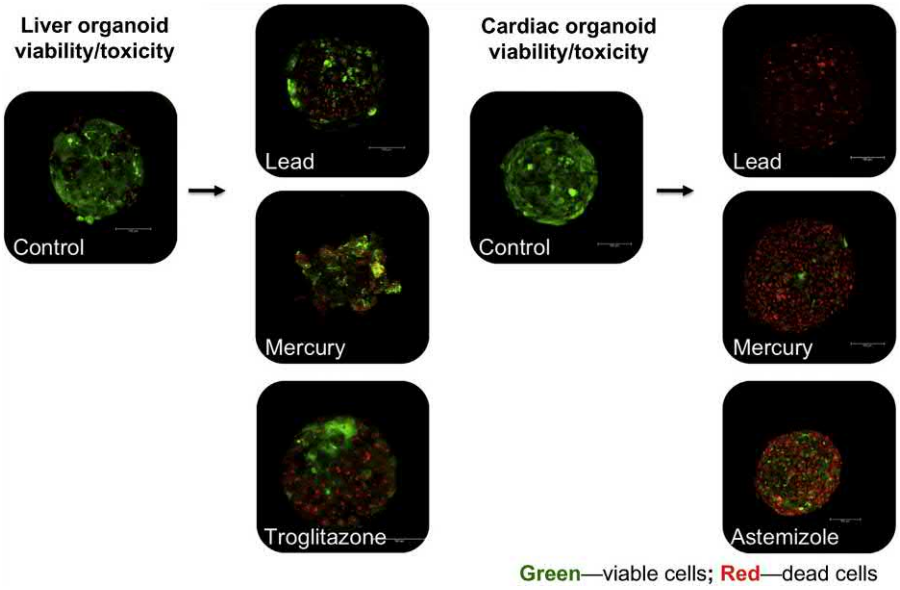

FIGURE 76.4 Drug and toxicology screening in liver and cardiac organoids. LIVE/DEAD stains show the presence of dead cells (*red*) after treatment with lead, mercury, troglitazone, or astemizole.

Additional disease modeling

Research on human pathologies other than cancer may also benefit from the capabilities of multiorganoid systems. There are many drugs and diseases that are known to cause inflammation. For example, large doses of the very common analgesic acetaminophen cause significant inflammation and toxicity in the liver. Other drugs, such as chemotherapeutics like bleomycin, cause inflammation, toxicity, and irreversible fibrosis in the lungs or other organs. In most of these cases, toxicity and apoptosis lead to the release of proinflammatory cytokines such as TNF-α and interleukin-1 into the circulation. These molecules can cause a series of downstream responses, including recruitment of inflammatory cells, activation of fibroblasts, and changes in vascular protein expression and permeability (Fig. 76.4C). Integrated multiorganoid model systems with integrated vasculatures can model responses across multiple organs and capture physiologically relevant effects that would be missed in single tissue models.

Cutting edge body-on-a-chip: the first highly functional multiorganoid systems

As described previously, there is a paucity of truly integrated multiorganoid platforms that are able to accurately model and test the complex responses to drugs, toxins, and disease across a range of human tissue types. However, progress is being made, primarily within the last several years.

The Ex vivo Console of Human Organoids platform

This system represents an advanced, modular multitissue integrated body-on-a-chip system for use in drug development, and toxicology screening. The multiorganoid body-on-a-chip platform is named Ex vivo Console of Human Organoids (ECHO). This platform was initially developed to include four engineered tissues representing liver, cardiac, vascular, and lung, which were integrated into a single system that provides real-time monitoring of physiological responses to toxic agents and pharmaceuticals.

Using the ECHO platform, a comprehensive set of data providing characterization of each organoid has been developed. In general, the 3D models are contained within platform microreactors using tissue type−specific supportive hydrogels. The result is an array of tissue equivalents suspended in a substrate containing tissue-specific ECM [36,91]. Liver models are fabricated using liver ECM-derived hydrogels that help maintain viability and function in vitro for over 4 weeks [91] Productions of key liver markers (e.g., albumin, multiple CYPs, epithelial cell−cell adhesion markers, dipeptidyl peptidase IV, and OST-α) been confirmed and are stable over a month. These models respond to toxins such as acetaminophen (APAP) at appropriate doses and in a dose-dependent manner. The models also respond to the clinical countermeasure for APAP intoxication, *N*-acetyl-L-cysteine by demonstrating reduced toxicity (Fig. 76.4A). Cardiac models also remain viable for 4 weeks, and beyond. These models demonstrate transport of fluorescent dye molecules among cells within the organoids, indicating a high degree of cell−cell communication. The constructs beat spontaneously and change their beating rates appropriately in response to a variety of drugs. These kinetics are captured using an onboard camera system [92,93] and custom software for analysis (Fig. 76.4B). In addition, an engineered vasculature has been incorporated into the

platform. The vessel model responds to agents such as histamine by disruption of the endothelial cell monolayer (Fig. 76.4C). This results in increased transendothelial transfer of larger MW molecules that are normally sequestered within the microfluidic circuit.

Several integrated, multitissue type studies have been performed using the ECHO platform. Experiments, including integrated liver, cardiac, and endothelial modules, have been performed in these microfluidic devices (Fig. 76.5A and B) under a common medium. These systems have demonstrated long-term viability, and produced multitissue responses to drugs in a manner that largely mimics the responses that would be expected in an experimental animal, or even in a human. For example, Fig. 76.5C describes the effects of propranolol and epinephrine on the cardiac model, both with and without integrated liver in the microfluidic circuit. Epinephrine induces an increase in beating rate in cardiomyocytes. Without liver, propranolol, a beta-blocker, blocks the β1- and β2-adrenergic receptors, preventing cardiac beat rate increase following epinephrine administration. However, in the integrated system that includes the liver model, propranolol is metabolized to an inactive form, resulting in the recovery of much of the epinephrine-induced increase in beat rate. To our knowledge, these experiments are the first interdependent in vitro multitissue type studies that

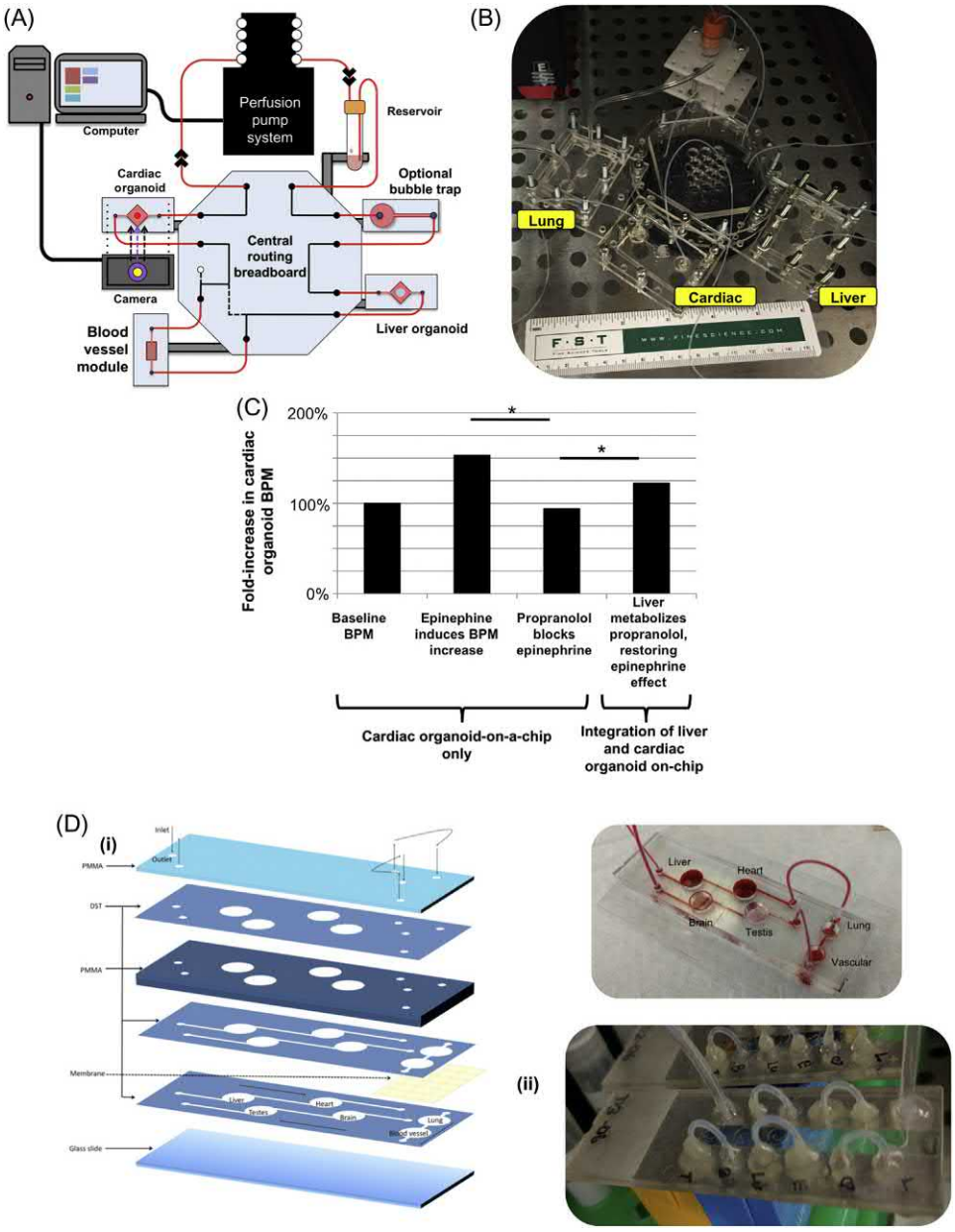

FIGURE 76.5 A multiorganoid body-on-a-chip. (A) A depiction of a liver, cardiac, and vascular organoid-containing body-on-a-chip platform. (B) A photograph of the three-organoid system. (C) Description of the effects of propranolol and epinephrine on cardiac organoids, with or without liver organoids, illustrating the importance of multiorganoid systems. Without liver, propranolol, a beta-blocker, blocks cardiac beating increases by epinephrine. However, with both organoids present, propranolol is metabolized by the liver organoid resulting in a measurable epinephrine-induced increase in beating rates. (D) (i and ii) Future body-on-a-chip platforms for increased capabilities for linking multiple organoids within a single circulatory system.

have been performed successfully in a single, integrated system.

The ECHO platform has also been used to screening drugs that were withdrawn from market due to unanticipated toxicities. Due to the lack of accurate models for predicting drug toxicity, many drugs have passed through preclinical studies and clinical trials, received FDA approval and remained on the commercial market for years, before being recalled for causing unanticipated toxic effects in humans. Approximately 90% of these drugs that have been removed from market result from toxicity in the liver and heart. A panel of these drugs has been tested in the ECHO platform. These include the drug troglitazone (Rezulin), an antidiabetic and antiinflammatory that was recalled for causing liver failure, and mibefradil, an ion channel blocker that was recalled for having fatal interactions with other drugs, including antibiotics. In the ECHO platform, troglitazone and mibefradil both demonstrated liver toxicity. The drug rofecoxib (Vioxx) is an NSAID that was recalled for causing serious vascular pathologies such as heart attack, stroke, skin reactions, and gastrointestinal bleeding. Astemizole, an antipsychotic that caused slowing of potassium channels, torsade de pointes, and QT prolongation; as well as terodiline, a drug for bladder incontinence that caused QT prolongation were tested in ECHO and showed functional changes and loss in viability among several cell types within the platform. The anticancer drug 5-FU and isoproterenol, a beta-adrenergic agonist, both of which are known to induce cardiac toxicity were evaluated in ECHO. Each of these drugs result in dose-dependent cell death within the cardiac constructs. Using the onboard camera, beating effects were observed to decrease in a dose-dependent manner as well. Notably, effects on beat kinetics were detected at doses well below the toxic threshold. This is an important point, as drugs withdrawn from the market for cardiac toxicity are generally not withdrawn for overt toxicity, but rather for causing arrhythmias.

Other body-on-a-chip programs

The ATHENA (Advanced Tissue-engineered Human Ectypal Network Analyzer) program has designed a miliscale multiorganoid system, and a program sponsored by the Defense Advanced Research Projects Agency (DARPA) has sponsored a 10-organoid project [94].

These projects have stressed aspects of microphysiological systems that are somewhat different than ECHO. Specifically, the ATHENA program, based out of Los Alamos National Laboratories, has developed a system comprised four organs—liver, heart, lung, and kidney [95]. These models are three orders of magnitude larger than the ECHO models. This scale allows for more relevant mechanical testing and the system fluid volume is sufficient for collecting samples that may be analyzed using standard clinical diagnostic equipment. The DARPA program, based out of Harvard's Wyss Institute, is working a collection of 10 models, including representations of endocrine, gastrointestinal, immune, musculoskeletal, and reproductive tissues [96].

In addition, the National Institutes of Health are supporting a major organ-on-a-chip program through the National Center for Advancing Translational Science, the National Institute for Biomedical Imaging and Bioengineering, the National Cancer Institute, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institute of Environmental Health Sciences, NIH Common Fund, and NIH Office of Research on Women's Health. The NIH initiative differs in that the funding is distributed among a variety of individual research laboratories and developing models for a wide range of tissue types [97]. The program stresses derivation of models from iPSCs, and many of the constructs developed by members of this program are nuanced and sophisticated. However, the potential for integrating these models into a multiorganoid has yet to be accomplished.

Organ-on-a-chip systems for personalized precision medicine

There is an increasing interest in developing in vitro models for personalized medicine. This is an unmet clinical need, as finding the most effect drug and dose for a specific patient is often a trial and error procedure. With personalized organoid models (Fig. 76.6A and B), therapies can be screened on a patient's own cells in a 3D tissue model system. For example, accurate prediction of a patient's tumor progression and response to therapy is one of the most challenging aspects of oncology. Prescribed treatments are often made based on the general success rate of a drug within a population, not on the specific response that may be expected within an individual patient. Recently, the concept of precision, or personalized, medicine has evolved to address these problems by using the patient's genetic profile to identify "drugable" targets for treatment [98–100]. However, in real-world practice, the results of this approach do not achieve the desired goals [101]. Following identification of key mutations through genetic profiling, physicians are still left with an array of drug options, with no concrete direction regarding potential side effects or the level of expected effectiveness. As such, there is a clear need to develop tools that can help predict the response of individual patients to drugs [102,103]. Current efforts are focused on the development of multiorganoid platforms that contain patient-specific tumor models in which therapeutic strategies are selected based on empirical results generated in the patient's on normal and malignant cells. Such systems

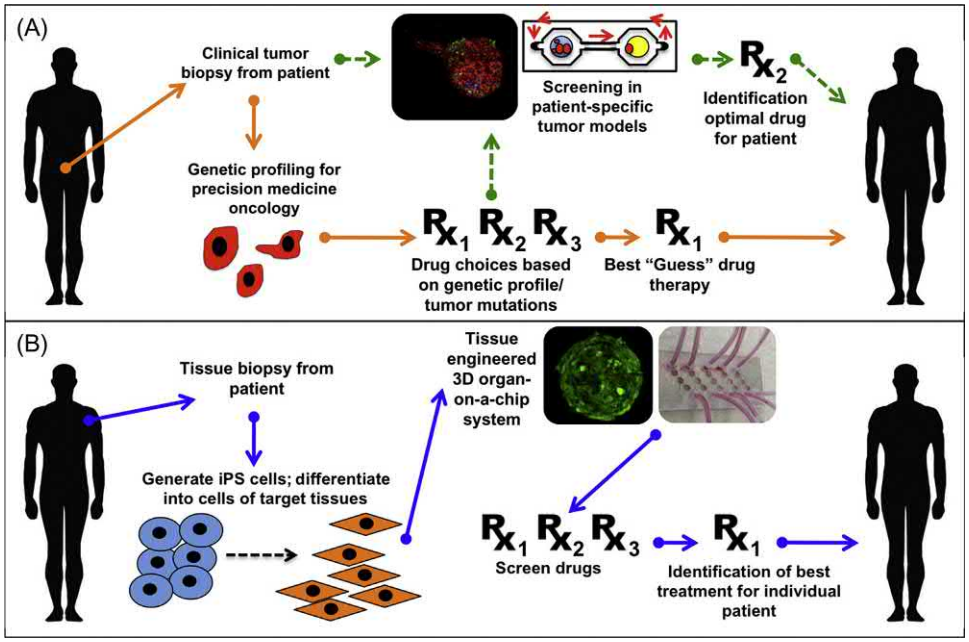

FIGURE 76.6 Employing biofabricated tissues in personalized medicine. (A) In personalized precision medicine for cancer patients (*red arrows*), currently a potential list of drugs are determined based on mutations found in the tumor genetic profile, from which best guess therapies are prescribed. In the future, cells from tumor biopsies could be used to create in vitro tumor models specific to a given patient (*green arrows*). Potentially effective drug therapies can then be screened in the models, thereby identifying the optimal drug therapy for that patient, both in terms safety and most effectiveness. (B) In genetic diseases, cells can be harvested from alternative tissues, such as skin, translated into induced pluripotent stem cells, differentiated into cells of the tissue of interest (e.g., lung or heart), and bioengineered into 3D organoids and organoid-on-a-chip systems, after which generic and genome-specific drug therapies can be screened for the original patient. *3D*, Three-dimensional.

may also indicate tissues to which metastatic cells may potentially migrate [84]. Taken together, these advanced 3D patient-specific models may allow clinicians to provide accurate prognoses regarding tumor progression and metastases and develop individually targeted treatment plans, personalized to each patient's tumor. As iPSC technology continues to evolve, it will likely be possible to generate an array of patient-specific tissue models that could provide information on the sensitivity of these tissues to specific treatment regimens. This would allow for the selection of an agent that spares healthy tissues while maximizing the killing of malignant cells. A final idea to consider is that the greatly reduced scale of these tumor models allows for the assessment of therapeutic efficacy against the small stem cell populations that are driving the bulk of the tumor, so long as these cells are captured within the model.

Conclusion and perspectives

While the benefits of multitissue type model systems are quite clear, there remain several challenges for their acceptance and widespread deployment for drug development and personalized medicine. Currently, most single cell type systems, do a respectable job at mimicking certain aspects of in vivo physiology and are quite amenable to high-throughput drug screening. The few single- and multitissue type models that include multiple cell types within each model provide a much better representation for human physiology, but they have not yet been optimized for high throughput [104]. As such, these more complex systems are best suited for evaluating drugs that are in the later stages of development. Many groups are actively developing strategies for multiorganoid systems and automating their production [14]. Significant reduction of size, automated fabrication, improved onboard biosensing and in-line diagnostic technology would greatly increase the throughput potential for multiorganoid platforms across all potential applications [92,93,105,106]. Another perceived challenge for the development of advanced, multiorganoid, platforms is the requirement of a common cell medium to support a wide variety of cell phenotypes. Typically, human primary cells and iPSC-derived cells require complex, highly specialized media formulations that are tailored to each specific cell type. Surprisingly, there is growing evidence that 3D cell constructs are intrinsically supported and much less reliant on complexed media supplements or serum. This has been demonstrated in the maintenance of a variety of cell types in human cancer models [18,84] using serum-free medium and customized hydrogen substrates [36,91,107]. Even more remarkable is the ECHO platform described earlier

in this chapter. In this platform, up to six organoid types, each containing up to five human primary cell types each have been maintained under a serum-free, common medium for at least a month with minimal loss in viability or function. It is likely that the constructs themselves produce the autocrine and paracrine factors that are required for long-term viability and function. The compact architecture, scant interstitial space between cells, and normal ECM proteins would allow these factors to become concentrated within each organoid and diminish reliance on exogenous factors delivered through the medium. Multiorganoid body-on-a-chip technology is advancing at a rapid pace and is likely to soon be deployed for drug screening [10]. These platforms have significant utility in many areas and will dramatically change the way that precision medicine, cancer modeling, and drug development are performed.

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Chapter 77

Monitoring and real-time control of tissue engineering systems

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Introduction

Tissue engineering (TE) systems (TESs) are defined as devices and accessories that enable the culture of engineered tissues for regenerative medical, diagnostic, or research purposes. These systems range in complexity, from a tissue culture plate in an incubator to TE bioreactor systems. This chapter will focus primarily on the monitoring and control in TE bioreactor systems. TE bioreactors are related to, but different from, industrial-scale fermenters. They are primarily used to mimic a physiological environment on a laboratory scale and to thus regulate temperature, pO_2, pCO_2, pH, nutrient concentrations, and osmotic environment. In some cases, they provide biologically relevant stimuli such as growth factors or mechanical input according to defined protocols.

Monitoring in a TES can be done at several levels: intracellular, extracellular/tissue, and environmental (Fig. 77.1). Cellular monitoring involves mostly fluorescence, bioluminescence, and other "imaging" modalities. Tissue monitoring may require microprobes (e.g., needle probes for O_2) and specialized hardware for measuring mechanical properties. Environmental monitoring can be very comprehensive and include most of the biomolecules, for example, oxygen, glucose, proteins, metabolites, and proteins in the culture medium.

Monitoring and control in TE bioreactors can be important for two main reasons (Fig. 77.2). The first is to ensure homeostasis of a selected parameter; this is common to most TES. Parameters that must be monitored in most cases include pH, pO_2, nutrient transport, waste removal, and temperature. Thermostatic control of the reactor temperature within defined limits is an elementary example. In such a case the variable that is monitored and the variable that is controlled are the same. In other cases the controlled variable can be used to indirectly manipulate the tissue. For example, glucose levels can be monitored and controlled to indirectly manipulate tissue growth.

The second reason for monitoring and control is the large (and still poorly understood) inter- and intra-donor variability in the performance of the cellular component. This variability affects critical TE parameters, such as the magnitude and time course of responses to growth factor treatment, tissue metabolic rates (and hence sensitivity to mass-transport limitations). This is particularly well-documented for mesenchymal stem cell (MSC)-based TE but also affects other cell types [1–3]. For cartilage TE, for example, this ultimately defines the composition, physical properties, and the timing of accrual of the extracellular matrix (ECM) [4]. It should be noted that failure in any one of these leads to an inadequate product [5,6].

A practical requirement of TE is to understand and predict process failures. Thus if issues are caught early during the development of the tissue, one can intervene either to address the issues or to decide to abandon a preparation to avoid implant failure and unnecessary costs. Nondestructive technologies with predictive skill are needed for monitoring engineered tissue during development. The monitoring should include key quality parameters for the end product, should be feasible in real- or near-real time, and should not compromise the prospects for implantation [6–8]. This is an interdisciplinary undertaking, which requires expertise in subject areas such as molecular and cell biology, biomedical, chemical, mechanical, and electrical engineering, advanced imaging, and computer modeling [6,8].

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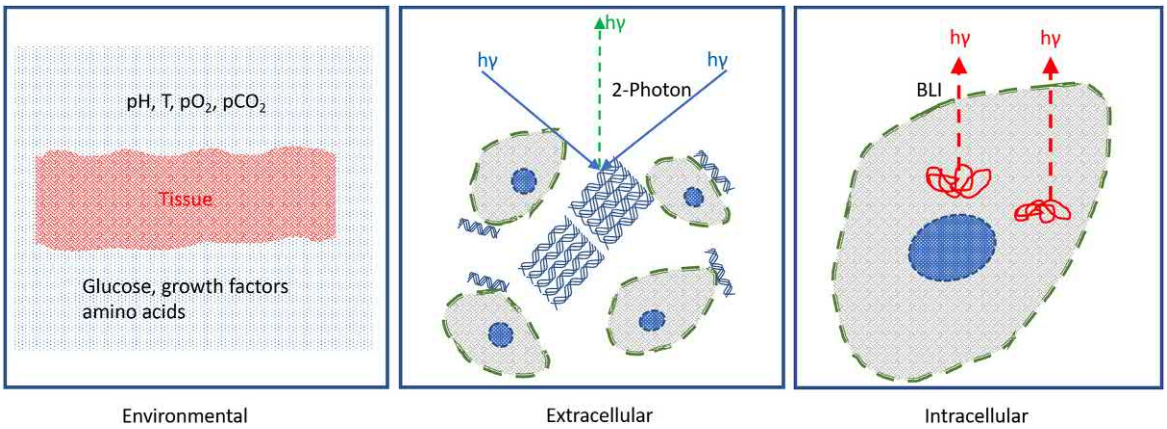

FIGURE 77.1 Levels of monitoring and control in TES. At the environmental level (*left panel*), pH, T, pO_2, pCO_2 are controlled along with monitoring of biomolecules glucose, growth factors, and amino acids. At the extracellular or tissue level (*center panel*), ECM deposition can be monitored using two-photon microscopy, ultrasound, or OCT. At the intracellular level (*right panel*), reporter-based systems can be used to monitor gene expression. *ECM*, Extracellular matrix; OCT, optical coherence tomography; *TES*, tissue engineering system.

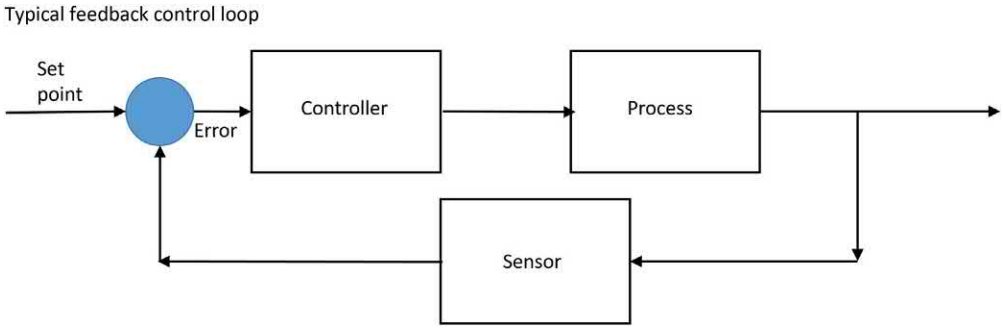

FIGURE 77.2 General flowchart for monitoring and control. In a conventional feedback control system the process variable (temperature as an example) is measured and compared to a set point, and a control action is performed to correct any error between the measurement and the set point. For many TES, monitoring of additional variables (compressive modulus) can be carried out without performing any action to correct any error between the measurement and the desired set point (release criteria). This is primarily due to two reasons: (1) we do not know the set point, (2) even if we do, we do not know what corrective action can be taken to address the error. *TES*, Tissue engineering system.

On the control side, currently, corrective actions based upon feedback for environmental parameters are probably the closest to being implemented. Thus temperature control is a mature technology, or for another simple example, glucose depletion can be counteracted by supplying more glucose (Fig. 77.2). Implementations will be discussed below. On the other hand, monitoring of emerging properties of the developing tissue may, at this point, only result in go/no-go decisions for the particular piece of tissue. In many cases, this is because we do not yet know what parameters to "tweak" to effect a correction.

A major challenge in monitoring and control of TES is that there is often no direct relationship between the parameters that are controlled, and the outcomes that are important for release criteria. This is partly due to a lack of knowledge regarding release criteria for any specific tissue, but more importantly due to inferior knowledge connecting tissue function to controllable parameters. For example, the compressive modulus of TE cartilage tissue may be related to glucose levels in the culture medium, but we do not yet understand the exact nature of the relationship. Consequently, at this point, we are not able to control the outcome of a TE exercise with respect to the release criteria, rather we focus on parameters that are considered critical to growth, differentiation, or maintenance of engineered tissue.

Current state-of-the-art

General environmental monitoring and real-time control

In most TES, there are key variables of the system that are always monitored and controlled. These include temperature, pH, and pO_2. The set points for these are based on standard (and sometimes historical) physiological values and not tissue-specific: for example, in a MSC-based cartilage TESs, temperature is set at 37°C though it

is well-known that in humans, both the bone marrow and knee joints have slightly reduced temperatures (~35°C) [9]. The list of controlled variables may include TES-specific variables such as mechanical strain for mechanically stimulated cartilage constructs (e.g., shear [10,11], compression [12], perfusion, hydrostatic load [13,14], or combinations thereof). While many other variables can be monitored, it is often difficult to control them. More importantly, currently, there is little understanding of how controlling such variables will affect the engineered tissue outcomes.

Temperature: Usually, a 37°C physiological set point is desired. This can be achieved by housing the TES in a commercial incubator or, in stand-alone systems, by using a heater and thermostatic control. In the former case, it should be noted that actuator systems associated with the TES (e.g., pumps, stirrers, etc.) can introduce excess heat if they are also enclosed in the incubator.

pH: In most TES a physiological pH set point of about 7.2−7.4 is desired, although for differentiating tissue, this value may be different. Culture medium pH is a good indicator of cell metabolism. Thus, in a perfusion bioreactor, pH is a qualitative proxy for nutrient availability/depletion. Most culture media have a pH indicator, for example, phenol red but quantitative measurement of pH requires sensors. pH sensors can be based on electrochemical or colorimetric methods [15]. As with every sensor-based system, fouling is a major concern, which limits long-term monitoring, especially in electrochemical sensors. In a typical bioreactor (fermenter) pH is controlled using acid/base injection. However, in a TES, pH control is more complicated and may be achieved by modulating buffered medium flow rate and to some extent by dissolved CO_2 content if a CO_2/bicarbonate buffer system is used.

Carbon dioxide: Control is primarily to maintain the buffering capacity of bicarbonate-based medium. If TES is housed in a commercial incubator, CO_2 produced by the tissue may not be a big issue. In self-contained bioreactors, CO_2 control should be built into the design using a dissolved CO_2 probe or a pH probe (as stated previously).

Oxygen: All mammalian tissues require oxygen for survival and function. The amount of oxygen required by the tissues, however, depends greatly on the state (e.g., differentiation) and type of the tissue. In vivo, oxygen delivery is primarily dependent on the transport resistance to oxygen and not on thermodynamic limitations (due to high oxygen carrying capacity). However, in vitro, oxygen transport to engineered tissues is greatly affected by both: the very low oxygen carrying capacity of culture medium and transport resistance of engineered tissues lacking a vascular network. Oxygen measurements of culture medium can easily be performed using oxygen probes. While oxygen reduction reaction−based probes (e.g., Clark electrode sensor) are popular, they are prone to error when oxygen levels are low. In such cases an optode-based sensor (e.g., ruthenium-based oxygen sensors) is superior and more accurate. Even though oxygen measurements using such probes are straight-forward, calculating oxygen uptake requirements of the tissue, is not. This is because often oxygen measurements are prone to leaks from atmospheric oxygen, and due to diffusional transport within and very near the tissue, oxygen values can drop precipitously near the tissue.

Control of O_2 can be achieved by changing the oxygen content in the culture medium (thermodynamic consideration) or by increasing medium flow rate (transport consideration). Both suffer from tissue-dependent limitations. In a metabolically active TES, even at the maximum thermodynamic limits, we may not be able to control oxygen content in culture medium for large tissues. This is because cells and tissues can be sensitive to shear caused by the flow conditions. In addition, medium washout can be a problem as paracrine signaling is considerably reduced under high flow conditions, though this can be ameliorated by recirculating the culture medium.

In TES the growth, differentiation or maintenance of the tissue is strongly dependent on the biochemical environment surrounding the cells and tissue. This biochemical environment comprises exogenous biomolecules such as oxygen, glucose, amino acids, proteins (e.g., growth factors), and endogenous cell-secreted biomolecules such as metabolites (e.g., lactate), amino acids, and proteins (e.g., ECMs and growth factors) biomolecules. While most researchers use a constant initial biochemical environment-defined exclusively by exogenous biomolecules, cell-based variability (e.g., donor-to-donor variability) and exposure to endogenous biomolecules produced during tissue culture have been shown to critically affect the dynamics of the TE process. This variability results in signature changes in metabolite or cytokine uptake/production rates, which can be measured through continuous monitoring of the biochemical environment. Later we discuss the various types of biomolecular measurements that can be beneficial for monitoring tissue quality.

Glucose is an important nutrient required by all tissues. In TES, only the glucose levels of the supply culture medium are set. These set values are not always determined as optimal based on the tissue requirements; rather, they are based on experiments done with small-scale tissue constructs in conditions that maybe quite different from the reactor conditions. In addition, for differentiating tissues, these supply medium levels may change with time as the metabolism of the differentiated cell phenotype is likely different from the progenitor cell metabolism. Glucose can be easily monitored through off-line measurements made using enzymatic electrochemical sensors [16]. Continuous glucose monitoring is improving

due to recent developments in glucose sensors (nonenzymatic) that have been a primary developed for diabetic monitoring/control [17,18].

Lactate is a key waste product of cellular metabolism, especially, glycolysis. Large levels of lactate in the cell culture medium signify oxygen-independent Adenosine Triphosphate (ATP) production. While this may be due to oxygen deficiency in the TES, it can also be an indication of dominant cellular metabolism. It has been shown that for chondrogenesis of human MSCs, lactate secretion is a good indicator of the quality of the tissue, even in the presence of oxygen [16]. Lactate measurements are obtained typically off-line using enzymatic and electrochemical/colorimetric methods [16]. Continuous lactate monitoring maybe possible using lactate oxidase–based sensors; however, long-term (days to weeks) use of such sensors in a TES has not been well-established.

Amino acids are key building blocks of proteins. Uptake and secretion of amino acids can be indicators of the degree of protein metabolism of the tissue. These can be crucial in connective TE as the primary functions of such tissue often require protein synthesis, often at levels considerably higher than the progenitor cell type (e.g., MSCs) from which the tissue is engineered. Amino acid monitoring requires tools such as LC/MS and therefore is done off-line using samples collected from the TES.

Uptake and secretion of proteins occur during the culture of any tissue construct. Proteins are used in the culture medium, typically, for signaling purposes. In the early days of tissue culture, fetal bovine serum's presence in culture medium was ubiquitous. However, due to concerns of disease transmission and serum batch-to-batch variability, more serum-free media are available now. An advantage of a serum-free defined culture medium is that it is easier to monitor proteins uptake/secretion. While proteins are not used to continuously monitor the tissue quality—primarily due to high cost of detection and measurement-secretion and uptake/binding of proteins can be very important in predicting tissue quality. For example, during epidermal differentiation in a skin TES, keratinocyte differentiation-associated protein (Kdap) secretion is a key indicator of differentiation [19]. Similarly, secreted phospholipases A2 (sPLA2) are found in the culture medium of differentiating keratinocytes [20].

While proteins in culture medium can be quantitated using ELISA or proteomic methods (e.g., mass spectrometry), reporter-based systems (see *Reporter Based gene expression Imaging*) can be used to monitor gene expression at the cellular level. In addition to ELISA, aptamer-based protein monitoring can be a powerful method, but it is not as widely available.

Tissue-level monitoring

At the tissue level, parameters such as ECM proteins, structure and organization, and mechanical properties of the tissue can be monitored. Strategies to controlling these parameters have not been developed yet.

Extracellular matrix structure

Noncontact, nondestructive insights into the structure of the ECM in tissues can be derived from acoustic parameters. In the example of cartilage, acoustic anisotropy of the tissue has been linked to the orientation of the ECM [21]. In cartilage TE, this anisotropy would be a desirable emerging property of the ECM and could be used to control, for example, directional mechanical stimulation.

Extracellular matrix amount/levels

Second harmonic generation using multiphoton microscopy allows the identification of ECM molecules, specifically collagen, in live unstained tissue in real-time, noninvasively and nondestructively [22,23]. Parameters such as collagen deposition per cell can be acquired. In combination with measurements of AA depletion (e.g., pro/hyp) in the medium, this could provide a useful feedback for controlling the medium composition.

In addition, in in vitro chondrogenesis, it is known that (particularly early on) a substantial fraction of newly synthesized ECM molecules are released into the culture medium [24,25]. This opens the opportunity for analysis of the synthesis profile [25].

Mechanical properties

For many TE constructs, matching the mechanical properties of the engineered tissue to the target tissue is a critical consideration. Techniques that evaluate the tissue in a nondestructive fashion could serve to evaluate tissue maturation and to inform decisions as to the maturity of the tissue, or to the trajectory of development (go/no-go decisions). Ultrasound elastography can be used to map strain fields through the tissue and, through modeling, can be used to determine local mechanical properties [26–32]. This can be valuable for determining whether the tissue is developing uniformly through its thickness. Other acoustic properties, such as speed of sound, reflection coefficient, frequency-dependent reflection coefficient, integrated reflection coefficient, ultrasonic roughness index, attenuation, and apparent integrated backscatter, can all be determined and provided for characterization of the tissue nondestructively [33–40].

Ultrasound

Ultrasound elastography is showing potential for evaluating the properties of TE cartilage noninvasively/nondestructively. In this case, light compression can be applied to the developing tissue in a bioreactor using an ultrasound transducer, and time shifts in returning signals (corresponding to internal displacements in the tissue) can be used to calculate internal strain fields [41].

Optical coherence tomography

Optical coherence tomography (OCT) has the potential to be a valuable technology for in vitro evaluation of engineered tissues [42]. Depth of penetration is small at a few mm but is sufficient for many some tissues. Axial resolution of OCT is better than ultrasound by one to two orders of magnitude. OCT has been used to evaluate cartilage arthroscopically in situ and in tissue explants; it is sensitive to ECM collagen orientation and fibrillation [42–51]. The speckle pattern in OCT images suggests that this approach could be coupled with elastography to determine internal deformation and mechanical properties of tissues [52].

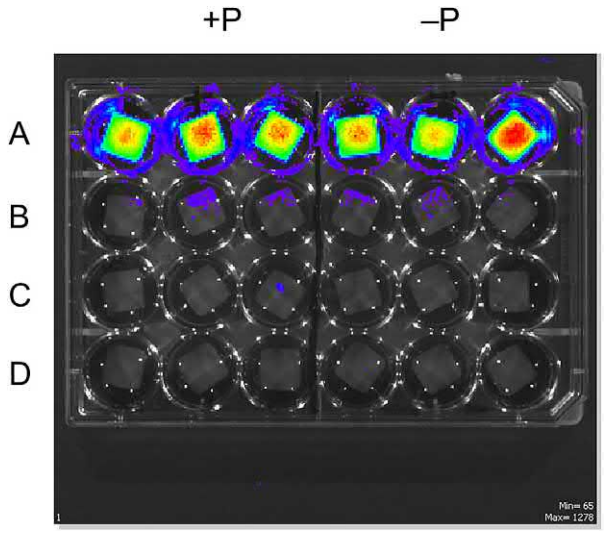

FIGURE 77.3 Example monitoring cells: non-invasively monitoring osteocalcin promoter activity in human MSCs using a luciferase-based reporter construct. Cells were seeded on different bone-implant substrate configurations *A–D*, with or without oxygen plasma etching (*+P, −P*). Two days after seeding, a decision can be made on which substrate to use going forward. Configuration A outperformed the other configurations for osteogenic differentiation support (cell numbers were identical across groups) plasma etching had very little effect. Constructs remain viable after this assay. *MSC*, Mesenchymal stem cell.

Cell-level monitoring

At the cell level, parameters such as gene expression or miRNA levels can be monitored. As discussed previously, strategies to controlling these parameters have not been developed yet.

Reporter-based gene expression imaging

Reporter gene–based measurements or bioluminescent imaging can be used to track molecular and cellular events (Fig. 77.3) [53]. In the context of monitoring and control in TES, event-specific or tissue-specific reporter gene constructs can be used. From a monitoring perspective the advantage of these approaches is that destructive sampling can be avoided and longitudinal imaging can be used to follow the evolution of the tissue over time. Imaging of the whole TE construct or sampling and quantitative analysis of the bioreactor medium (in the case of secreted reporters) can be done [54]. At this point, no automated controls have been implemented to respond to fluctuations in this type of signal in real time. However, capturing such changes can lead to the decision to proceed, or to modify or discontinue growth of particular samples.

In the example of tissue cartilage, reporter gene constructs with Col2 promoter-driven luciferase have been developed [55] to track chondrogenic differentiation [56]. Similarly, the promoter for the transcription factor Sox 9, a master regulator of MSC chondrogenic differentiation, and aggrecan, an ECM component, have been developed into reporter systems for use during TE chondrogenesis [54,56–60]. During the later stages of cartilage formation the expression of proteases and proteinases [e.g., matrix metallopeptidases (MMPs) and tissue inhibitors of MMPs (TIMPs)] that are involved in cartilage remodeling can signal differentiation past the optimal point for articular cartilage [61]. MMP-13 promoter-driven reporters for imaging MMP-13 are available [62–65], as are reporters driven by the TIMP3 promoter [66,67].

An alternate, potentially much simpler approach is to use noncoding RNAs (e.g., miRNA)—responsive reporter constructs to track pathway regulation [68,69].

Tissue-specific

Below, we describe some tissue-specific considerations for control and monitoring.

Cartilage monitoring and real-time control

Cartilage TESs involve bioreactor culture of constructs larger than a few millimeters. Fig. 77.4 shows a disk-shaped cartilage construct (12 mm diameter × 3 mm thickness) cultured in a perfusion bioreactor. The bioreactor consists of a transparent membrane made of fluorinated ethylene propylene membrane that allows free exchange of oxygen and carbon dioxide between the

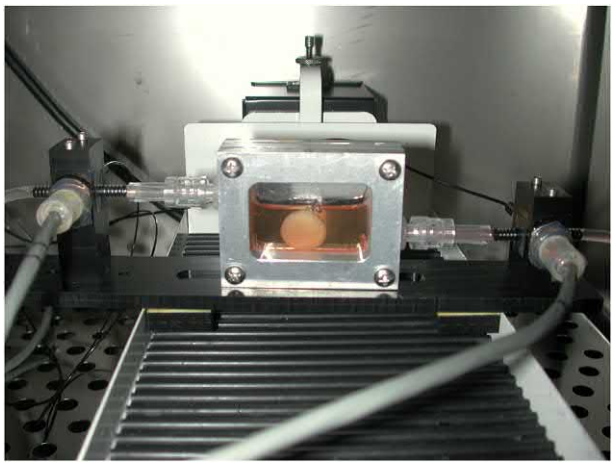

FIGURE 77.4 Cartilage environment monitoring and control. Tissue engineered cartilage construct in a perfusion bioreactor culture. Fiber optic pH sensors sense the color change of the pH indicator dye phenol red in the medium between the inlet (*right*) and the outlet (*left*) of the bioreactor.

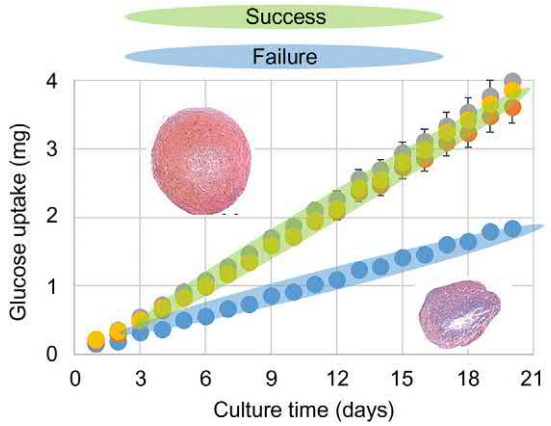

FIGURE 77.5 Glucose uptake as a proxy go/no-go decision maker. Glucose uptake measured over time for different culture conditions. Higher glucose uptake led to improved cartilage ECM as determined by the Safranin-O staining of the histological sections (*inserts*). Even as early as day 3, glucose uptake of a failed culture condition is significantly lower than that of the successful conditions. *ECM*, Extracellular matrix.

culture medium and the incubator environment in which the reactor is housed. In this bioreactor, pH was monitored using probes at the inlet and outlet using a colorimetric method.

Glucose monitoring in an MSC-based cartilage system can be used to make decisions on success/failure of the culture. Fig. 77.5 shows glucose uptake measured over time for different culture conditions. Higher glucose uptake led to improved cartilage ECM as determined by the Safranin-O staining of the histological sections (inserts). While control of glucose maybe achieved by increased flow or higher glucose concentration in the medium, continuous monitoring is still essential to make go/no-go decisions.

Skin

Skin equivalents, a.k.a., reconstructed epidermis are emerging for use in drug testing and possibly for burn coverage. Large-scale skin engineering will require bioreactors. Early attempts at growing keratinocytes on perfusion bioreactors were largely run without tissue-derived feedback [70]. Standard environmental parameters, temperature, CO_2, and medium flow rate are controlled as described previously. It should be noted that most of the skin culture in the literature is done at constant 37°C. It is, however, known that skin temperature is generally lower than that and experiences circadian and exertion-dependent fluctuations [71]. It may be useful to mimic these conditions in skin TES going forward. There will also likely be the need to culture the skin constructs at an air−liquid interface, as this is required for the maturation of the epithelial layer that recreate the barrier function.

For vascularized skin graft constructs, perfusion pressure is controlled. A physiological pressure profile including systolic and diastolic pressures can be provided [72]. Emergent barrier function of the skin equivalent can be monitored nondestructively during tissue maturation by impedance spectroscopy [73].

Although not traditional TE, in vitro expansion of skin grafts to increase the available coverage is being developed. Here tension is applied to skin explants and, stretch magnitude and rate are monitored, and controlled increases in area in the range of 110%−180% have been achieved [74,75].

Cartilage and skin are probably the most investigated tissues for TE and regenerative medicine. Other tissues are being considered; some relevant markers at the cell, tissue, and environmental levels are shown in Table 77.1.

Concluding remarks

TES comprises a complex ecosystem of cells, scaffolds, and medium to produce a piece of tissue for study or implantation. Active monitoring and control are indispensable for optimal tissue production. The monitoring side includes traditional cell culture environment variables that not only apply to most cell types and tissues but also to a wide variety of variables such as mechanical stimulation that are specific to the tissue of interest or its intended function. On the control side, homeostasis or programmed profiles of the common environmental variables are well understood, but for the more tissue-specific variables, the current state-of-the-art may only allow for

TABLE 77.1 Tissue type and monitored/controlled variables.

TES type	Intracellular	Extracellular/tissue level		Environmental	Other	References
		ECM proteins	Mechanical			
Cartilage	Sox9, RUNX2	Type 2 collagen	Compressive and shear loading	Physiological pH, T, pO_2[a], pCO_2		
Skin	Involucrin, keratin profiles, transglutaminase	Type 1 collagen, fibronectin	Tensile loading	Physiological pH, T, pO2, pCO2	Air–liquid Culture	
Bone	Osteocalcin, RUNX2	Type 1 collagen, alkaline phosphatase	Compressive loading, tensile loading	Physiological pH, T, pO_2, pCO_2		
Heart valves	MMPs and TIMPs, α-SMA	Type 1 collagen, elastin	Stretch, flexure, flow, pressure cycling	Physiological pH, T, pO_2, pCO_2		[76,77]
Cardiac tissue	α-MHC, MLC-2V, Nkx2.5, NCX-1, KDR/Flk-1	Type 1 collagen	Flow through, contraction, and relaxation	Physiological pH, T, pO_2, pCO_2	Vascularization	[78]
Trachea	Sox9	Type 2 collagen	Hydrodynamic stimulus	Physiological pH, T, pO_2, pCO_2		[79]
Lung	α-SMA	Type 1 and 4 collagen, elastin	Contraction and relaxation	Separate alveolar and blood environments	Microfluidic	[80]
Liver	P450 expression	Type 1 collagen		Lactate, albumin, urea, pO_2 is critical		[81–84]
Intestine	ZO-1, E-cadherin, Villin		Cyclic deformation	Separate oxygen deficient luminal epithelial side and normoxic subepithelial side		[85,86]

ECM, Extracellular matrix; *MMP*, matrix metallopeptidases; *TES*, tissue engineering system; *TIMP*, tissue inhibitors of matrix metallopeptidases.
[a]*Normoxia/hypoxia.*

go/no-go decisions on the entire TE protocol under study. Acquisition, databasing, and dissemination of more tissue responses to TE parameters will likely allow for more granular responses in the future.

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Chapter 78

Biomanufacturing for regenerative medicine

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This chapter entitled will cover the current landscape of biomanufacturing where we will highlight current technology and areas where biomanufacturing is established and biomanufacturing is seeking to mature. We will also consider current workflows for biomanufacturing and what are some of the top challenges are that need to be addressed. Platform technologies that are enabling biomanufacturing will be highlighted, as well as covering the regulatory challenges and looking toward the future.

Current landscape of biomanufacturing

Biomanufacturing encompasses the scale-up of processes that permit the consistent production of biological products at a commercial scale. Our specific focus in this chapter is biomanufacturing for cell therapies and tissue engineering applications fundamental to regenerative medicine. Current technology for biomanufacturing is still being developed, but we can focus on areas that are well established and will review some of them later. Many of these other areas of manufacturing have processes that could be adapted to biomanufacturing and assist with bringing this relatively young field to a higher level of maturity within a short period of time. There is no need to reinvent the wheel. Rather, rapid progress can be made by adapting and reconfiguring these manufacturing processes from other fields to biomanufacturing.

For example, automobile manufacturing is well developed. We would like to highlight an automobile production process management system from HITACHI [1] and draw parallels to biomanufacturing where many of these processes could be adapted. The sequencer accurately drafts production sequence plans for automobile manufacturing. Similar production sequence plans could be generated for biomanufacturing clinical products. Airlocation controls the storage of complete automobile yards on a real-time basis. This parallels having on-demand biomanufactured products in a warehouse or tissue bank that could communicate in real-time between the manufacturers, hospitals, clinicians, and patients when a clinical product is available. POP controls the production instructions and result collection for the manufacturing sublines. A parallel with biomanufacturing is that this system could be used to collect real-time data from the biomanufacturing of a clinical product to ensure product quality attributes. Unit processing controls the casting, forging, and processing processes for unit products in a lot production system where this could aid biomanufacturing processes by layering in a quality control process for raw materials that could be used in manufacturing the clinical product. Unit assembly controls the assembly and inspection process for unit products in a mixed-low production system. A parallel here to biomanufacturing is that this system could provide quality assurance as the product is assembled, expanded, matured, and packaged for delivery/storage. NXAUTO is a software package that supports the automobile manufacturing process. This could be assimilated into the biomanufacturing management system as a software package that would integrate the different biomanufacturing processes while also providing in-line sensing quality controls in real time to optimize the manufacturing process to produce a clinical product with the quality attributes of identity, strength, quality, purity, and potency needed to ensure safety and efficacy.

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Antibody manufacturing is also well developed. Monocolonal antibodies are a major class of biopharmaceutical products. Traditional large-scale manufacturing processes using stirred tank bioreactors for the growth of Chinese hamster ovary cells often require large initial investments and ongoing production costs. Molecular farming is an alternative method for using plants for large-scale production of biopharmaceuticals. Molecular farming has been demonstrated to be safe, scalable, and capable of reducing manufacturing costs [2]. This may be a technology that can be applied to biomanufacturing of regenerative medicine–based products. For instance, one potential cost advantage could be achieved by accumulating recombinant proteins in seeds for long-term protein stability without the need of the cold chain. Interested readers who would like to learn more about advantages and disadvantages of molecular farming are encouraged to consult this review on the current state-of-the-art in plant-based antibody production systems [2].

Vaccine manufacturing is advancing with new production methods that can produce vaccines more efficiently [3]. These new production methods can take processes that used to take 6–36 months to produce, package, and deliver high-quality vaccines and reduce their production time significantly. One solution that GE Healthcare Life Sciences is implementing to speed production is the development of turnkey facilities, out-of-the-box manufacturing platforms. Companies are also finding ways to accelerate vaccine development in emerging economies. One example is from Millipore Sigma, approach of which is to support companies in emerging economies in various ways such as assisting with streamlining manufacturing processes or sharing expertise. This act of corporate social responsibility will help in advancing the global vaccine industry. This lesson of corporate social responsibility can also be applied to biomanufacturing where regenerative medicine products need to be available also for emerging economies.

Chimeric antigen receptor (CAR) T is a cell therapy approach to treat cancer. While we will not address the entire clinical manufacturing process of CAR T cells here, interested readers should seek these reviews [4,5]. CAR T cell therapy is quite effective in treating acute lympoblastic leukemia [6–8]. However, manufacturing clinical-grade CAR T cells under current good manufacturing practices (cGMPs) to enable widespread use of this therapy is still a challenge and one that biomanufacturing will share as well. There is need to develop platform technologies that are efficient and cost manageable to support commercialization. These platform technologies will be implemented along the entire manufacturing process of CAR Ts, which generally speaking include the following steps: (1) apheresis collection, (2) apheresis product wash/fractionation, (3) T-cell selection, (4) T-cell activation, (5) gene transfer, (6) T-cell expansion, (7) T-cell formulation, (8) T-cell cryopreservation, and (9) infusion to patient [4]. In addition to these manufacturing processes there is also an in-process and quality control testing performed throughout the entire manufacturing process.

The field would like to take the manufacturing processes developed for the areas previously mentioned and apply them to cell therapies, personalized medicine, and tissue engineering. In the following section, we will consider current workflows for biomanufacturing for regenerative medicine that would benefit greatly from the more developed manufacturing processes highlighted earlier for automobile manufacturing, for instance.

Highlighting current workflows for biomanufacturing

In this section, we will briefly highlight three common workflows envisioned for biomanufacturing clinical products (Table 78.1). These include (1) allogeneic, (2) autologous, and (3) xenogenic workflows. Many perspective articles have been written that go into additional detail on these workflows, and interested readers are encouraged to consult these reviews [9–14]. An allogeneic workflow would consist of taking a donor's cells (tissue acquisition), isolating those cells, expanding them, harvesting them, bioprinting or seeding them on a scaffold (for a tissue-engineered product), packaging the cells (or tissue-engineered product), and then shipping or cryopreserving the cells (or tissue-engineered product) before they are administered to the patient. Along this workflow is integrated in-line testing to assist with ensuring quality, safety, and efficacy of the cells or tissue-engineered product. Autologous workflows are similar but would use patient's own cells to produce the cell therapy or tissue-engineered product. This will call for a manufacturing process that can be mobile and decentralized and set up at primary care facilities or will incorporate a rapid two-direction shipping model for raw material and final product. Xenogenic workflows are similar to allogeneic workflows but will engineer nonhuman cells or tissue raw materials to be manufactured into a clinical cell therapy or tissue-engineered product.

Current challenges in biomanufacturing for regenerative medicine

The current challenges in biomanufacturing for regenerative medicine are many, but we would like to highlight some of the top challenges, which are summarized in Table 78.2. These include the high costs of biomanufacturing a clinical product. One study done in the United

TABLE 78.1 Manufacturing workflows for regenerative medicine.

	Allogeneic workflows	Autologous workflows	Xenogenic workflows
Overview	This workflow enables true off-the-shelf products where the cell- or TERM-based product is derived from a human source that is not the patient	This is a patient centric workflow that develops a cell- or TERM-based product from a patient's own cells	This workflow enables off-the-shelf products where the cell- or TERM-based product is derived from a nonhuman source
Approach	Cells or tissues are acquired, isolated, expanded, harvested, and bioprinted	Cells or tissues are acquired, isolated, expanded, harvested, and bioprinted	Cells or tissues are acquired, isolated, expanded, harvested, and bioprinted
Therapeutic potential	Off-the-shelf products will be favorable for commercial potential. Potential concerns with immune rejection will need to be addressed	Novel manufacturing processes will need to be developed to take these patient derived therapies and expand them in parallel to a commercial size that is cost manageable	Xenogenic products will be off-the-shelf and favorable for commercial potential. Additional tests will be needed to ensure product safety since cells will be genetically engineered and from a nonhuman source

TERM, Tissue-engineered regenerative medicine.

TABLE 78.2 Current challenges in biomanufacturing and potential solutions.

Current challenge	Description of challenge	Potential solutions
High cost	Very limited cost data to begin with, but the costs for cell therapy and tissue-engineered organs to manufacture under cGMP conditions for a clinical product are very high and will be difficult to make these treatments widely available	Development of universal, defined media for cell manufacturing. Development of a universal bioink for 3D bioprinting
Insufficient automation	Many of the biomanufacturing processes are performed manually and are not fully automated	Develop fully automated processes for biomanufacturing that can be used with multiple cell types for cell therapies or tissue-engineered products
Underdeveloped QC	There is need especially for in-line sensing quality control that is nondestructive	Develop nondestructive in-line sensing technology that can be integrated into automated processes to ensure quality attributes of the biomanufactured clinical product
Lack of sufficient standardization	One specific gap is the standardization of ancillary materials for biomanufacturing	Development of standardized criteria and documentation to ensure consistency and quality for all ancillary materials used in biomanufacturing processes
Lack of modular systems	Currently there are no modular systems that can be configured for biomanufacturing	Develop modular universal bioreactors for expanding and maturing cells and bioengineered tissues and organs

cGMP, Current good manufacturing practices; *QC*, quality control.

Kingdom provided cost data for a stem cell–based tissue-engineered airway transplant ranging from $174,420 to $740,500 for three patients treated [15]. This study provides some of the first cost data really documenting the expense behind bioengineering an organ. Some solutions to combat these high costs are listed in Table 78.2 and are also addressed in a perspective article cited here [16]. Insufficient automation is another challenge where many biomanufacturing processes are still performed manually. Another challenge is having underdeveloped quality control metrics and in-line sensing systems. There is specific need for real-time data that could be gathered throughout

the manufacturing process and done so in a nondestructive manner. Standardization is another gap. In particular, there is great need to standardize ancillary materials that are used in biomanufacturing. Ancillary materials are the raw materials used during the biomanufacturing process for the cell therapy or tissue-engineered product, but they are not intended to be part of the final product. Many considerations have been made on ancillary materials and how they could affect the clinical product and patient safety [17]. There is also a gap in technology in creating configurable modular systems for biomanufacturing. These could be modular bioreactor systems for cell expansion or any other subsystems that could accommodate different cell types and biomanufacturing processes. One could imagine plug-and-play modules that could be connected together to build out an end-to-end biomanufacturing process for any cell or tissue-engineered product. In the next section, we will consider some platform technologies that will enable biomanufacturing.

Current platform technologies enabling biomanufacturing

Media development for biomanufacturing is a platform technology that is being advanced. The Food and Drug Administration (FDA) provided a nice perspective on mesenchymal stem cell (MSC)-based characterization for clinical trials [18], where they reviewed four parameters that could affect product characteristics in manufacturing MSCs, which included (1) fetal bovine serum (FBS), (2) atmospheric oxygen, (3) cryopreservation of the final product, and (4) cell banking (working or master cell banks). Developing a defined media that avoids the use of FBS would address one of these critical parameters which the FDA remarks over 80% of regulatory submissions on investigational new drug (IND) applications for MSCs use FBS in their manufacturing process [18]. Removing FBS from media formulations will increase the consistency of the product and reduce variance from different manufacturing lots. Interested readers who want to learn more about considerations for serum replacements should consult this review [19]. Some commercial suppliers are developing serum-free media and also an industry-driven consortium effort is making progress on developing a serum-free defined media to support clinical cell manufacturing [20].

Another platform technology that will enable biomanufacturing is bioprinting. 3D printing technology has the potential to decrease production time by enabling same day production and shipping [21]. Potentially, a product that would normally take a month to go through design modification phase now only takes 7 days. As an analogous case study, for Ford to create a brand new mold every time they wanted to test a new part of their engines takes 4−6 months and tens of thousands of dollars. Ford can now produce these molds in just 4 days with costs amounting to only $4000. Perhaps we will find ways for 3D printing technology to provide similar benefits to biomanufacturing with enabling innovation to occur more rapidly with introduction of new bioprinted scaffolds or bioprinted organoids or 3D printing novel bioreactors or 3D printing connectors that could all optimize performance and function of the new regenerative medicine clinical product. Essentially 3D printing manufacturing facilities could innovate new technologies on the fly and test them out in a rapid fashion. Another advance in 3D bioprinting is the development of tunable bioinks that can be used across different bioprinting platforms. Many commercial entities are working on designing these bioinks, and an industry-driven consortium effort is also making progress on the development of a tunable bioink for 3D printing that would have applications in both tissue engineering and also personalized medicine [20].

Next-generation biomanufacturing platform systems do not currently exist in their entity, but there are components that do exist. These biomanufacturing platforms consist of scale-up systems, quality control systems, and automation. For scale-up systems, these would include bioreactors that could be used to expand different cells of interest under controlled and monitored conditions. They could also be envisioned to accommodate maturation of cells and priming of tissues and bioengineered organs to be ready for physiological demands. For a more comprehensive review of the current and future states of bioreactors, readers are encouraged to consult this review [22]. Quality control systems need to be used throughout the entire manufacturing process and can provide a report card on the quality attributes of the cells, tissues, or bioengineered organs being biomanufactured. These quality control systems could incorporate real-time monitoring of pH, lactate, glucose, and other attributes as well as designing omic (genomics, proteomics, and metabolomics) assays for more comprehensive profiling. There is some progress toward automation for cell therapies [22,23]. The robotic technology to fully automate biomanufacturing processes currently exists, but defining and standardizing these processes for each clinical product is still a challenge. Also automation will have to be thought about differently based on allogeneic (off-the-shelf products) versus autologous (patient specific) workflows. Artificial intelligence (AI) can also be integrated into automation and will be one of the most disruptive platform technologies for biomanufacturing. AI could be used for smart maintenance to reduce unplanned downtime and maximize productivity. It could also be used to alert manufacturing teams of emerging production faults that could lead to quality

issues with a product. AI could also be used as a personal assistant to perform routine functions and provide teams with sound recommendations [24].

Collectively, these platform technologies will all enable biomanufacturing. In the following section, we will consider regulatory challenges for biomanufacturing.

Regulatory challenges for biomanufacturing

In their structural complexity and heterogeneous mechanisms of action, tissue-engineered products represent a paradigm shift from conventional drugs and devices. While they may be constructed of multiple cellular, biochemical, and structural components, their function is more like a graft or organ transplant than a drug or device. And rather than a daily dose to alleviate symptoms, a tissue-engineered organ often promises a cure in one dose.

Thus a primary challenge for biomanufacturing of tissue-engineered products is one of regulatory categorizations. Internationally, the International Conference on Harmonization, the World Health Organization, and various national and international agencies or coalitions such as European Union legislate or guide regulatory compliance and categorization. In the United States, and addressed primarily in this chapter, the US FDA regulates drugs, biologics, and devices differently. A tissue-engineered organ, which may contain cells, growth factors, and a biomaterial scaffold, is considered a combination product, consisting of a biologic and drug component (cells and/or growth factors, for example) and device (biomaterial scaffold). Which FDA center regulates the product and whether an IND or investigational device exemption (IDE) application is required depends on the primary mode of action of the product: whether the device portion or the drug portion has the most important role in intended efficacy (for more information, see Ref. [25]). Generally speaking, tissue-engineered therapies have required INDs and clinical trials according to the Public Health Service (PHS) Act section 351. Meanwhile, certain minimally manipulated, homologous use products may not require INDs or clinical trials according to PHS Act 361. In additional to PHS regulation, most biologics are also regulated under the Federal Food, Drug, and Cosmetic Act [FD&C Act, Code of Federal Regulations Title 21, or 21 CFR (Codes of Federal Regulation)] since they are categorized as drugs.

The FDA's Center for Biologics Evaluation and Research regulates biologics, which include everything from vaccines to tissue-engineered organs. Meanwhile, the FDA's Center for Drug Evaluation and Research regulates other biological products more biochemical in nature such as monoclonal antibodies and growth factors used for therapeutic applications (for more information, see Ref. [26]). Thus the regulatory pathway for a specific tissue-engineered product may not always be straightforward, and final categorization may take communication with the FDA to determine.

The 21st Century Cures Act of 2016 [27] was designed to help accelerate moving from innovation to the clinic more efficiently. This included a new regenerative medicine advanced therapy (RMAT) designation that allowed for an expedited development pathway if (1) it is a non-361 regenerative medicine therapy; (2) it is intended to treat a serious life-threatening disease or condition; and (3) "preliminary clinical evidence" indicates potential to address an unmet medical need for the target condition [28]. The FDA released a suite of four guidance documents in 2017 to clarify its position on minimally manipulated, homologous use biologics and RMAT designation (see the first four guidance documents in the list next).

The tissue therapy biomanufacturing community was hoping the abovementioned developments in regulatory pathway would include a broadening of therapeutics that would fall under PHS Act 361 or the benefits of RMAT designation, thus lightening the regulatory and financial pathways to market. This has generally not been the case for the most innovative tissue-engineered products, as the homologous use definition is very narrowly defined for 361 classification and prior clinical evidence is required for RMAT designation. First in human applications do not apply.

A major focus of the FDA in recent IND filings has been the requirement for biocompatibility testing. Every new application or change in formulation adds about 50,000−100,000 USD and about 6−18 months to the IND approval process. While identical materials, or materials from different vendors, are already approved, there is little tolerance for justification of equivalence instead of testing in a climate that is highly risk-averse and lawsuit-prone.

While there are numerous clinical trials in progress, to date there is only one stem cell therapy approved by the FDA: the use of cord blood for hematopoietic regeneration; bone marrow is also used for similar applications but is not currently regulated by the FDA [29]. As of 2015, about 11 regenerative medicine or tissue-engineered products had reached the market approved by the FDA; see Table 78.3 [30]. Of the biologics and cell-based devices, three used autologous and three used allogeneic cell sources. The majority of tissue-engineered organs use autologous cells seeded on a synthetic or biological scaffold material. In 2016 the FDA approved the first tissue-engineered autologous cellularized scaffold product for the repair of cartilage defects in the knee of adult patients: Maci, by Vericel Corporation, is composed of cultured chondrocytes on a porcine collagen membrane

TABLE 78.3 Regenerative medicine products approved as of 2015 [30].

Product	Category	Biological agent/application	Biologics source
lViv	Biologics	Fibroblasts/improving nasolabial fold appearance	Autologous
Carticel	Biologics	Chondrocytes/cartilage defects from acute or repetitive trauma	Autologous
Apligraf, GINTUIT	Biologics	Cultured keratinocytes and fibroblasts in bovine collagen/topical mucogingival conditions, leg and diabetic foot ulcers	Allogeneic
Cord blood	Biologics	Hematopoietic stem and progenitor cells/hematopoietic and immunological reconstitution	Allogeneic
Dermagraft	Cell-based medical devices	Fibroblasts/diabetic foot ulcer	Allogeneic
Celution	Cell-based medical devices	Cell extraction/adipose stem cells	Autologous
GEM 125	Biopharmaceuticals	PDGF-BB, tricalcium phosphate/periodontal defects	Allogeneic
Regranex	Biopharmaceuticals	PDGF-BB/lower extremity diabetic ulcers	Allogeneic
Infuse; Inductos	Biopharmaceuticals	BMP-2/tibia fracture; lower spine fusion	Allogeneic
Osteogenic protein-1	Biopharmaceuticals	BMP-7/tibia nonunion	Allogeneic

[31]. The Armed Forces Institute of Regenerative Medicine (AFIRM) grants awarded by the department of defense since about 2011 have committed more than 75M USD for military population–targeted regenerative medicine applications, including extremity repair, craniomaxillofacial reconstruction, skin injury and burn repair, composite vascular allotransplantation and immunomodulation, and genitourinary repair and lower abdomen reconstruction [32]. With the limited number of approved tissue-engineered products on the market, this was an ambitious push to move more of these into the investigational sphere with potential for broader application in traumatic injury and degenerative condition repair for the general population in the near future.

The hope is that with continued innovation and FDA guidance, these few products that have been approved will set precedence for the safety and efficacy of regenerative medicine products and the pathways to investigational approval and market approval will be accelerated.

Food and Drug Administration guidance documents

In the United States, the CFRs are the laws to follow for regulatory compliance. To help apply these in tissue engineering and regenerative medicine products, the FDA offers various nonbinding guidance documents. Where compliance requires adherence to standards or specifications set by other organizations such as the International Organization for Standardization (ISO), United States Pharmacopeia, the PHS, and the National Institute of Standards and Technology, those documents will generally cite those sources. Specifically which CFRs and guidance documents will apply depends on the categorization and phase of clinical trial or licensure, as briefly discussed previously. Representative CFRs and guidance documents that may apply in tissue engineering and regenerative medicine are discussed in Table 78.4.

These regulations begin to provide the framework on how biomanufactured clinical products will be regulated. From these important documents, biomanufacturing processes can be developed to ensure a smooth transition through the regulatory approval process. In Table 78.5 a list of representative nonbinding guidance documents provided by the FDA is given.

These important guidance documents provided by the FDA serve to provide direction and recommendations for industry in numerous critical areas, including considerations for human cells, tissues, and cellular and tissue-based products; expedited programs for regenerative medicine therapies for serious conditions; and how to properly control and validate electronic recordkeeping systems. The more direction that is provided, the better industry can develop biomanufacturing processes in compliance with federal regulations to enable mass production of safe and effective regenerative medicine–based clinical products.

TABLE 78.4 Representative US regulations governing current good manufacturing practices (cGMPs), good tissue practices (GTPs), or related tissue processing requirements.

- 21 CFR 312: Investigational New Drug Application
- 21 CFR 812: Investigational Device Exemptions
- 9 CFR 113.53: Requirements for ingredients of animal origin used for production of biologics
- 21 CFR 4: cGMP requirements applicable to combination products (and see 21 CFR 3)
- 21 CFR 11: Electronic Records; Electronic Signatures
- 21 CFR 210: cGMP in Manufacturing, Processing, Packing, or Holding of Drugs; General
- 21 CFR 211: cGMP for Finished Pharmaceuticals
- 21 CFR Parts 600 through 680: Other applicable regulations for biological products
- 21 CFR 820: Quality System Regulation
- 21 CFR Part 1271: Human cell, tissue, and cellular and tissue-based products (HCT/Ps)
- Section 351 of the PHS Act (42 U.S.C. 262): Drugs, devices, and/or biological products requiring clinical trials
- Section 361 of the PHS Act (42 U.S.C. 264): Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps) meeting certain minimally manipulated and homologous use criteria

CFR, Codes of Federal Regulation; *PHS*, Public Health Service.

TABLE 78.5 Representative guidance documents available to assist in compliance to the US regulations governing current good manufacturing practices (cGMPs), good tissue practices (GTPs), or related tissue processing requirements.

Representative nonbinding guidance documents provided by the FDA

- Evaluation of Devices Used With Regenerative Medicine Advanced Therapies; February 2019
- Guidance for Industry and FDA Staff: Regulatory Considerations for Human Cells, Tissues, and Cellular and Tissue-Based Products: Minimal Manipulation and Homologous Use. Issued November 2017; Updated December 2017
- Guidance for Industry: Same Surgical Procedure Exception under 21 CFR 1271.15(b): Questions and Answers Regarding the Scope of the Exception, November 2017
- Draft Guidance for Industry: Evaluation of Devices Used With Regenerative Medicine Advanced Therapies. November 2017
- Draft Guidance for Industry: Expedited Programs for Regenerative Medicine Therapies for Serious Conditions. November 2017
- Draft Guidance for Industry and FDA Staff: Technical Considerations for Additive Manufactured Devices. May 10, 2016
- Draft Guidance for Industry and FDA Staff: Medical Devices Containing Materials Derived from Animal Sources (Except for In Vitro Diagnostic Devices), January 2014.
- Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products, November 2013
- Guidance for Industry: Preparation of IDEs and INDs for Products Intended to Repair or Replace Knee Cartilage, December 2012
- Guidance for Industry: CGTP and Additional Requirements for Manufacturers of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps), December 2011
- Guidance for Industry: cGMP for Phase 1 Investigational Drugs, July 2008.
- Guidance for Industry: Regulation of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps) Small Entity Compliance Guide, August 2007
- Guidance for Industry: Computerized Systems Used in Clinical Investigations, May 2007
- Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—cGMP, September 2004
- Guidance for Industry: Part 11, Electronic Records; Electronic Signatures—Scope and Application, August 2003

CFR, Codes of Federal Regulation; *CGTP*, current good tissue practice *FDA*, Food and Drug Administration; *IDE*, investigational device exemption.

Creating standards

Quite often, compliance in regenerative medicine, particularly tissue engineering, is an interpretive art since the CFRs and guidance documents do not have a special category for those technologies. Cells are still considered "drugs" and "biologics" can be anything from vaccines to tissue-engineered bladders, so determining improperly which regulations apply can lead down expensive and time-consuming wrong directions when applying related similar prior experience or the first apparently related guidance document. Some areas where greater guidance for tissue engineering would be helpful are discussed, as the problems seen in one setting are not directly related to another.

Stem cell–based therapies have a history of safety concerns, primarily with direct mutagenesis or genetic plasticity. Both human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) have history of teratoma formation; MSCs may be associated with undesired differentiation, and their otherwise advantageous immune suppressive and angiogenesis properties may also indirectly promote metastasis [33]. The FDA has concerns that immortalized cell substrates in vaccine production could cause cancer in recipients [34,35]. The concern logically extends to tissue-engineered products. However, it is difficult to apply a direct genetic test for abnormalities since autologous, somatic cell populations may already naturally contain varying levels of donor age–dependent aneuploidies in a wide range of abundance; for example, nullisomy Y may occur naturally in men in about 10%–73% of cells [36–39]. While some karyotyping studies showed increases in gross aneuploidy in culture from early to late passages [40], others showed decreases, indicating selection against chromosomal aberrations in culture rather than proliferation [41]. This, combined with similar results in other studies, indicates that there is little evidence that karyotypic drift in culture of MSCs or differentiated somatic cells contributes to mutagenesis as seen in hESCs and iPSCs, and supports that these abnormal cells are typically reduced or eliminated in culture [42–44]. Since tissue-engineered products generally use differentiation pathway–dedicated stem cells or somatic cells, the mutagenesis risk appears largely mitigated in these products. Nevertheless, the clinical significance of the karyotypic variations previously noted when delivered in an allograft or autologous cell or tissue therapy product is widely unknown and unstudied. There is currently no requirement by the FDA to conduct karyotypic analysis of tissue-engineered products or as part of a panel of suggested safety testing for general biologics produced by aseptic processing. Some additional guidance and updated information by the FDA in this regard specific to the various categories of tissue engineering and cell therapy applications would be helpful.

Virtually all INDs are returned with an instruction to develop potency assays prior to later phase trials. This can be difficult since the elements defining the potency of an organ (or even a cell) are complex and heterogeneous. Some advanced guidance in what has constituted adequate potency assays for tissue-engineered products would be helpful.

Some common reagents and components are generally considered to be averse to inclusion in cell-based final products. Phenol red is an example; while there is no specific restriction to its use, and phenol red is used as a pharmaceutical, some prior work has indicated that it should be removed, and so organizations go to great lengths (and great cost) to remove it from all cell expansion products. Some clear guidance or standards on safe amounts of phenol red and other common reagents would be helpful.

The future: envisioned advanced biomanufacturing

Biomanufacturing of tissue-engineered organs or organ subcomponents is currently a very manual, hands-on process. The ideal would be completely closed, automated systems. The typical product requires suspending a biomaterial scaffold in a bioreactor, sterilizing it, then reopening the system to rehydrate it, seed cells on it, and mature it. While parts of the process can be performed in closed systems, and technology exists, there currently exist no commercially available systems to either fully automate or fully close production of a tissue-engineered organ from cell expansion through final product formulation.

Closed-modular biomanufacturing systems

As a replacement for the most common system (an open clean room with manual operators), the fully closed organ production system can be developed with or without automation. First, a system without automation can be a modular system that connects specialized ISO5, HEPA-filtered enclosures for all incubations and critical equipment, such as cell sorters and centrifuges. Manipulations can be performed manually via glove boxes or mechanical pass-through manipulators or robotics. The product would be moved to various locations within the modular system for specific parts of the process, but it would not leave the enclosure until final product was prepared and in its clinical delivery containment and closure vessel. The entire system would be sterilized by ethylene oxide or another system such as plasma phase H_2O_2 (Sterrad.

In this model, cell expansion systems could use conventional 2D flasks or could incorporate 3D technologies, including carrier beads or automated fiber systems such as the Terumo Quantum. The system, like standard clean rooms and equipment, would have to be monitored and controlled for temperature, humidity, CO_2, ongoing functionality of equipment when not directly observed by operators, etc. Consumables would need to be loaded into the system prior to sterilization (if compatible with resterilization), and all systems and consumables would need to be validated for sterilization method. This system would be relatively easy to construct with existing technology, given enough financial and time commitment to design and build for one dedicated purpose at a time.

As a second-tier technology, a fully automated system could be developed. These systems could be designed to place all critical components within it and then press the

green button to produce a tissue-engineered organ in a few weeks. While all technology exists to do this even today, the integration of technology has not been attempted. For example, the Quantum cell expansion system exists, but it does not inherently monitor critical culture condition indicators such as glucose and lactate levels. The fully automated system would need to not only do that but also make decisions based on the readings, and carry out the actions necessary for expansion and production. For this to work the process itself would have to be highly standardized and characterized, and the automation system would need to integrate chemistry systems, optical monitoring, digital image transformation with software algorithm interpretation, macro- and microfluidic controls, robotics, and automation of any specialized equipment such as cell sorters.

Off-the-shelf products

A critical step in the scale-up of tissue-engineered organs is mass production. This is most easily accomplished by a decrease in the personalized medicine nature of these products through standardization of more universally compatible products. This can be done through immune-modulated allograft-based products, or production of a suite of off-the-shelf products compatible with a spectrum of human leucocyte antigen (HLA) types. Entire organs, or critical components, can be cryopreserved, thawed, reconstituted, combined, and prepared as final products, ideally with the automated systems described earlier, and ideally within a few hours of a physician placing the prescription. Only in one of these off-the-shelf modes can these products truly meet the high demand and perpetual shortage of organ donations available for transplant.

Preservation advances

Currently, long-term storage requires cryopreservation or lyophilization. Both of these can drastically change the physical structure of tissue-engineered organs, scaffolds, cells, or other subcomponents. Future cryopreservation or stabilized cold storage (nonfrozen, liquid storage) would ideally be DMSO free and should maintain the inherent shapes and structures of the tissue-engineered organs or subcomponents. Researchers have studied the biochemical hibernation mechanisms of larger mammals and smaller organisms that can survive under desiccation or freezing conditions for seasons or even years; these natural coping and preservation mechanisms will someday be harnessed to allow long-term preservation of large tissue-engineered organs or organ subcomponents.

Synthetic biology advances

As chemistry, rheology, and biocompatibility advances continue to be made in bioprinting, better, more customized scaffolds and scaffold materials are currently being developed. The groundwork for making on-demand scaffolds has already been prepared. Organ structures, bones, and support structures can be bioprinted from patient MRI or CT scans. To make this a reality for patient treatment, standardization of production methods and development of more robust, sterile, and reliable bioprinting technology are needed.

A key requirement of 3D biological structures is vascularization. Current technology employing decellularization of entire organ structures retains blood vessel structure at the capillary level, awaiting autologous recellularization. These structures could be mass produced from standard forms or could be replicated on demand by bioprinting. Alternatively, advances in angiogenesis using specialized MSCs could be employed as a hybrid technology with bulk scaffold structures.

Cell banking advances

For mass production and off-the-shelf products, banking and storage systems will need to be optimized. In addition to improvement of biological material stabilization for long-term storage noted above, banking systems will need to be updated to handle the large amount of banking that will be needed and to fit the custom, large organ or tissue shapes that need preservation. The standard vials used in cell banking will no longer work. Bag systems will need to be widely employed to maintain system closure. Importantly, reliable and automated, optically or RF coded labeling and retrieval systems will need to be employed. The cryopreservation freezers themselves will need to be redesigned to geometrically better handle the volume of materials in unusual sizes and shapes—rectangular boxes in round freezers may no longer be practical.

Medical applications for biomanufacturing in regenerative medicine

We next wanted to highlight case examples for medical applications for biomanufacturing. To do this, we have highlighted current products in the cell therapy and tissue engineering clinical trial pathway at Wake Forest Institute for Regenerative Medicine (WFIRM) (Table 78.6).

These case examples highlight where the next-generation of health care is going to provide personalized medicine, on-demand tissues and organs, and even innovative treatments for our wounded warriors.

The AFIRM grants took a major leap forward in providing a prime source of funding for tissue engineering

TABLE 78.6 Case examples of regenerative medicine therapies advancing at an academic clinical center.

Regenerative medicine therapies in clinical trial pipeline at WFIRM
• Tissue-engineered corpus cavernosum for repair of damaged penile tissue
• Tissue-engineered urethra for repair of strictures or other damage
• Tissue-engineered internal anal sphincters for repair of fecal incontinence
• Tissue-engineered vagina for restoration of normal function from congenital defects or traumatic injury
• Tissue-engineered bladder for repair and reconstruction following damaged or disease
• Volumetric muscle repair and replacement from injury or disease, beginning with tissue-engineered muscle repair for cleft lip
• Would healing using amniotic membrane powder
• Testicular tissue banking prior to ablative chemo therapy or radiation therapy for future restoration of fertility
• Muscle progenitor cell implant for repair of stress urinary incontinence
• Bioprinted skin
• Tissue-engineered intestine
• Bioprinted nasal septum
• Fetal placental and amniotic fluid stem cell master cell banks to be used for a broad spectrum of autologous applications, including wound healing, gene therapy for hemophilia, necrotizing enterocolitis, erectile dysfunction, abdominal adhesions following surgery, and neonatal lung damage

WFIRM, Wake Forest Institute for Regenerative Medicine.

applications geared toward repair of battlefield injuries at a time when there were no tissue-engineered organs on the market. The grants provided invaluable funding and advancements to the field of tissue engineering and have moved a half dozen or more tissue-engineered organs from the lab bench to the doorstep of first in human clinical trials at WFIRM alone. A prime focus of WFIRM's tissue-engineered products has been genitourinary repair, a key need in an era when battlefield armor and medicine have been able to save so many lives, while roadside bombs leave targeted lower limb and pelvic floor injuries in high numbers in young men and women in prime marriage and child bearing age. The restoration of their ability to bear children, in addition to the cosmetic and functional repair for normal sexual, urinary and fecal function in general, restores these soldiers not only in their physical functionality, but also in their self-confidence and sense of wholeness as well.

While battlefield injuries will unfortunately always be a reality and a potent motivator for public funding to honor this specific and deserving population, the natural extension of this work is that the general population will always have a need for these technologies as well. Automobile, gunshot, construction, and untold other types of accidents leave similar injuries in average citizens every day. Moreover, the potential to offer off-the-shelf engineered organs to replace the need for cadaveric organ transplants is one of the holy grails of the future of medicine. Many of these technologies exist in incipient form today and can be brought to fruition through continued major financial investment from interested organizations; by participation by the regulatory agencies in guiding the regulatory framework to streamline development and clinical testing of these products; and through investment of time and resources by companies willing to develop the enabling technologies for automation, closed systems, and mass production.

Space exploration

This technology is often called "science fiction become science fact" by people who realize how advanced the technology currently is and how close it is to making major changes in the face of medicine. This technology may be absolutely necessary as the next phase of science fiction moves closer to science fact: extended space exploration. Plans are in development in the United States for long-term colonies to be established on both the Moon and Mars. While many people may be willing to take on the risk for the sake of adventure, many others will be less willing to sacrifice themselves for the cause by dying of treatable conditions while on these colonies until all medical capabilities and populations are developed to replicate all medical support present on the Earth. Tissue engineering technologies offer one mechanism to treat major medical conditions with limited population resources, such as cadaveric donors for organ transplants. Bioprinters and modular tissue engineering labs could be placed both on space stations and Moon and Mars colonies. All of the battlefield injury repairs currently under development for tissue-engineered therapies would be candidates for the types of injuries one may face in the rugged early phases of colony development. And the longer people spend on those colonies, the more likely the population will face the needs for organ transplantation, including kidney, lung, heart, and bladder.

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Part Twentytwo

Clinical experience

Chapter 79

Tissue-engineered skin products

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Introduction

Skin tissue engineering was perceived as the simplest tissue engineering application and was the first to be explored. The culture of fibroblasts, derived from skin biopsies, was established early. The cells were comparatively easy to grow and were used to develop many basic tissue culture techniques. The culture of keratinocytes was achieved by Rheinwald and Green in the late 1970s [1], and using a different system, by Liu and Karasek [2]. Skin biology was second only to immunology in the application of the techniques of molecular and cell biology to medical problems, which has led to great advances in the understanding of the physiology of skin. Examples of methods that have been applied include the use of classical techniques of molecular and cell biology and transgenic animals.

The skin acts as a barrier between internal structures and the external environment. In the 1980s it was realized that the skin, in addition to acting as a barrier, also has significant interaction with both the innate and the adaptive immune systems [3,4]. It is thus much more than a passive barrier and actively recruits defensive mechanisms to protect against infection, colonization by microorganisms, and other external noxious entities.

With a long history of development, tissue-engineered skin substitutes have achieved market exposure and have some of the most extensive experiences with the application of such products in a therapeutic setting. From its inception in 1979 [5], engineered skin was seen as potentially a clinical product and trials started in the 1980s. Preclinical testing was inaugurated and demonstrated that fibroblasts were apparently not rejected and persisted for a considerable period in experimental animals [6–8]. This led to human clinical trials in the late 1980s and early 1990s. Tissue-engineered skin products first gained regulatory approval and appeared on the market in 1997. The first was TransCyte (Advanced Tissue Sciences, which was a nonviable burn product produced by tissue engineering techniques). It was followed by Apligraf (Organogenesis) in 1998, the first tissue-engineered, viable, organotypic product, Dermagraft in 2000 and Orcell in 2001. At the same time, many applications of such products for in vitro testing applications and models of skin physiology were developed.

Much of the discussion in this article is based on experience with Dermagraft and TransCyte. Both products have a similar basis in growth of fibroblasts on three-dimensional (3D) scaffolds. The systems, however, differ substantially in detail.

Dermagraft is grown on 2 in. × 3 in. sheets of a knitted polylactide/glycolide scaffold under static conditions in a bag bioreactor. Following seeding, the cultures are refed with medium every few days until harvest after about 2 weeks. Initially, the fibroblasts proliferate rapidly, much as in monolayer, but as they become confluent, at about 8–10 days, they lay down increasing amounts of extracellular matrix and form a dermis-like structure. At harvest, medium is replaced with cryoprotectant, the bioreactor is welded closed without exposure of the product to the environment, boxed, and frozen under controlled conditions. Dermagraft is stored below −65°C for up to 6 months. During the first month, sterility and analytical testing is performed and the paperwork completed to allow release of the product by the quality assurance department. On thawing, which is performed by the end user, the cells of Dermagraft show 50%–80% viability.

TransCyte was grown using a similar process on two 5 in. by 7 in. sheets of a knitted nylon (nondegradable) scaffold with a silastic backing in a hard bioreactor with a continuous flow of medium. This system has advantages in the ease of taking samples of the medium for analysis and in slightly superior cell growth but it adds considerably to the complexity of the system. At harvest the cultures were rinsed, the bioreactors sealed from the system by welding, packaged, and frozen without any precautions

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taken to maintain viability so that the final product was not alive.

The 3D structures formed by the cells under these conditions consist of cells embedded in extracellular matrix that they, themselves, have secreted. It comprises a complex series of molecules. The process of secretion may be thought of as formation of a foreign body capsule in vitro.

Types of therapeutic tissue-engineered skin products

The development of the manufacture of tissue-engineered skin has taken two fundamentally different approaches using alternative methods for the 3D culture of fibroblasts and a third that has intermediate characteristics. One is the use of fibroblasts suspended in collagen gel, in which the gel acts as a substrate for the growth of keratinocytes and forms a structure that has some resemblance to human skin. The second involves culture on a 3D nonbiologically derived, polymeric scaffold. Fibroblasts are responsive to signals from their environment and respond in quite different ways to the two types of culture. In a third system, fibroblasts are grown on a collagen sponge in serum-containing medium. Since collagen adsorbs fibronectin and vitronectin, this system initially shows cell adhesion to both collagen and fibronectin and vitronectin and the cultures show intermediate properties. A fourth approach, using 3D printing methods, will not be discussed as it is a fundamentally different approach that is not scaleable using the concepts discussed here.

In collagen gel suspension, fibroblasts are surrounded by collagen and respond through $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, which are collagen receptors, by becoming quiescent and nonproliferative [9]. Characteristically, the collagen gel is contracted by the fibroblasts to less than 10% of its original volume in about 20 hours, through the $\alpha_1\beta_1$ integrin. At the same time, many genes, notably those for collagen type I and proliferation, are repressed [10] and the cells show mechanosensitivity [11], also mediated through the $\alpha_1\beta_1$ integrin [9]. If the gels are mechanically stressed, usually by preventing contraction with a ring, many genes, including collagen type I and proliferation related genes, are somewhat induced relative to contracting gel fibroblasts, although not to the level of monolayer fibroblasts [11]. The cells also show a substantial upregulation of genes associated with inflammation such as the genes for IL-1, IL-6, and cyclooxygenase [12].

When grown on scaffolds, fibroblasts proliferate rapidly and then deposit large amounts of extracellular matrix. Initially, the conditions on the scaffold are not dissimilar from monolayer culture. The scaffold adsorbs proteins from serum, which is a component of the medium still used for fibroblast culture. The adsorbed proteins include fibronectin and vitronectin as in monolayer culture [13]. However, as the cells reach higher densities and the fibroblasts cease to proliferate, they proceed to lay down extracellular matrix at rates approaching their own weight per day. The matrix is loose connective tissue, much like the provisional matrix of granulation tissue. The cells are clearly very active and induce several genes, among the most notable of which are neutrophil chemoattractant chemokines, CXCL1, CXCL5, CXCL6, and CXCL8 (gro-α, ENA-78, GCP-2, and IL-8, respectively). They also induce the expression of IL-6 and IL-11. The conclusion from these observations is that the fibroblasts act to recruit neutrophil granulocyte components of the innate immune system, which is important in responding to the most likely type of environmental insult to which the dermis is liable, physical injury followed by bacterial colonization.

Components of tissue-engineered skin grafts as related to function

Scaffold

Several types of scaffold have been employed for tissue-engineered skin implants. They include collagen gels, knitted polylactate/glycolate (PLGA) or nylon fabrics, and collagen sponges. As discussed earlier, the scaffold has profound effects on gene expression and the function of the fibroblasts.

The Dermagraft and TransCyte processes used scaffolds obtained as finished commercial products from other companies, which already had regulatory approval. While this simplified the development of the products, it ultimately proved an expensive decision. A solution to this is to obtain a second source of raw material that is not a finished product and establish its equivalence.

A further point here is that, while the use of already approved products as scaffolds may be an easy initial path, the available materials may not be ideal for the particular application envisaged. There remains a need to expand the range of scaffolds available for commercial tissue engineering. As an example, enzymatically, rather than spontaneously, degrading scaffolds may be more suitable for tissue engineering applications, allowing control of the degradation process.

An interesting direction is the development of scaffolds incorporating a vascular capability. Revascularization of skin substitutes is a limitation in their application and the ability to be perfused by the host circulatory system would be a major advance. Scaffold with this type of capability may be made by decellularizing a perfused tissue or organ and then using the remaining extracellular matrix structure as a scaffold [14].

Keratinocytes

Keratinocytes generate the impervious surface of the skin (the stratum corneum) and also have been thought of as having a major role in defense against microbial colonization as activators of immune responses. As a consequence, keratinocytes have been included either alone or in combination with other components in many skin implants [15]. Their role in forming a physical barrier is important, but they also have the ability to produce antimicrobial peptides, such as β-defensin, psoriasin, and cathelicidin [16,17] and a wide variety of cytokines capable of activating immune responses [18,19]. The epidermis contains antigen presenting cells that are capable of activating T lymphocytes under suitable conditions to both cell-mediated and humoral adaptive responses. This is largely a function of Langerhans cells, but it is possible that the keratinocytes also take part under special conditions. It has been argued that the application of a keratinocyte-containing bag to a wound, permeable to proteins but not to cells, may be beneficial without incorporation of the cells into the patient (United States Patent 5972332).

It is notable that the keratinocytes show a higher expression of the fibroblast stimulating platelet-derived growth factor (PDGF) A chain gene than do fibroblasts. This may constitute part of the secretion of reciprocal paracrine growth factors by the epidermis and the dermis [20]. Such a pathway has been described by Fusenig, involving IL-1α secretion by keratinocytes, which stimulates secretion of FGF-7 (keratinocyte growth factor 1) and granulocyte-macrophage stimulating factor that promotes keratinocyte proliferation [21]. Such interactions may well be of importance during wound repair when proliferation of both types of cells is important [22].

In practice, allogeneic keratinocytes in skin implants appear to remain for some weeks and then disappear. This has been attributed to immunological rejection, but this is not clear as keratinocytes, cultured to the numbers required for a skin implant, consist almost entirely of transiently amplifying cells that follow a differentiation pathway that leads to a modified form of apoptosis. They may, thus, be expected to be lost from the implant without the intervention of the immune system.

An approach that has been explored for increasing the coverage and survival of grafted epidermis is to mix allogeneic with autologous keratinocytes. This permits a comparatively small number of autologous keratinocytes, without extensive expansion, to cover a large area [23]. The allogeneic cells provide initial coverage, while the autologous population, which retains stem-like cells, provides a persistent surface. Most of the allogeneic cells are eventually lost, but the epidermis remains.

An alternative application of keratinocytes to wounds by spraying has been explored. The cells survive the experience, and the technique provides a very simple and convenient way of applying them [24]. It is possible to envisage a system spraying largely allogeneic keratinocytes together with a fresh autologous keratinocyte suspension isolated intraoperatively from skin or scalp [25].

Fibroblasts

Fibroblasts are the major producers of extracellular matrix, discussed in the following section. They are not antigen presenting cells and have been regarded as not having a major role in interactions with the immune system. However, their ability to secrete neutrophil attractant chemokines, CXCL-1, CXCL-4, CXCL-5, and CXCL-8, and the cytokines IL-6 and IL-11, discussed later, suggests that they may have a more important role in activating innate immune responses.

Fibroblasts do not appear to produce significant quantities of defensins, cathelicidin, or other antimicrobial peptides. Their major antimicrobial activity appears to be through recruiting neutrophils.

Extracellular matrix

Fibroblasts express a large array of extracellular matrix genes. Transcripts giving the 30 highest signals on Illumina expression arrays are listed in Table 79.1. Expressed genes represent collagen types 1, 3, 5, 6; proteoglycans (decorin, lumican); thrombospondins; fibulins; fibronectin; tenascin C; metalloprotease inhibitors; lysyl oxidase; tissue factor inhibitor; and secreted protein, acidic, cysteine-rich. They also include matrix-bound growth factors, such as FGF-2 and TGF-β. The matrix includes multiple kinds of integrin ligands, including those for integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_1$, $\alpha_7\beta_1$, $\alpha_6\beta_4$, and proteins that are known to modify adhesion. It provides complex stimulation that, since it is secreted by fibroblasts, is presumably appropriate for cell migration. In systems where fibroblasts are cast in collagen gels, the initial signal is through the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin and that remains the dominant stimulus until the cells elaborate other signaling molecules such as $\alpha_6\beta_4$.

Subcutaneous fat

Subcutaneous fat has received little attention thus far in the engineering of skin. However, it has been shown to undergo major structural changes during the hair cycle in the mouse and is a highly vascular organ. Fat-derived stem cells have been used successfully in the production of skin substitutes and may provide advantages over fibroblasts [26].

TABLE 79.1 Extracellular matrix proteins secreted by fibroblasts in three-dimensional culture, as a percentage of the protein giving the highest signal on an Illumina expression array [secreted protein, acidic, cysteine-rich (SPARC)].

Extracellular matrix protein	Signal as percent of SPARC (%)
Collagen, type I, alpha 1	18.83
Collagen, type I, alpha 2	36.51
Collagen, type III, alpha 1	30.96
Collagen, type IV, alpha 1	4.67
Collagen, type V, alpha 1	12.54
Collagen, type V, alpha 2	11.85
Collagen, type VI, alpha 1	17.60
Collagen, type VI, alpha 2	5.66
Collagen, type VI, alpha 3	16.85
Collagen, type VIII, alpha 1	6.61
Collagen, type XVI, alpha 1	6.20
Decorin	15.13
Biglycan	5.92
Lumican	42.19
SPARC (osteonectin)	100.00
Testican	7.49
Spondin 2	7.83
Fibrillin 1	10.24
Elastin microfibril interfacer 1	5.72
Microfibrillar-associated protein 2	0.29
Microfibrillar-associated protein 4	8.34
Fibulin 1	0.27
Fibulin 2	7.77
Fibulin 2	7.77
Epidermal growth factor-containing fibulin-like extracellular matrix protein 2	6.86
Fibronectin 1	0.39
Tenascin C (hexabrachion)	10.98
Thrombospondin 1	30.53
Thrombospondin 2	8.50
Tissue factor pathway inhibitor 2	21.16
Laminin, gamma 1	12.91

Components of the immune system

Inclusion of Langerhans cells has been accomplished in test systems but has not been applied to therapeutic products. As they are antigen presenting cells, they would have to be autologous (see later), and thus their inclusion would not be suitable for a scaleable manufacturing system,

Melanocytes

Other cells of the epidermis have been incorporated into test systems and have also been considered for transplant into epidermal tissue—engineered therapeutic products. An example is the inclusion of melanocytes as a treatment for vitiligo, which is a clinical problem in countries such as India. Systems, including melanocytes, have been used

extensively in test systems for ultraviolet radiation protective preparations.

Adnexal structures

The self-assembly of hair follicles and their use as a therapeutic product is discussed later in this chapter. Inclusion of sweat glands has been explored, but they have not yet been included in tissue-engineered constructs. The early fear that patients treated with cultured keratinocyte allografts might have problems with temperature control has not eventuated.

Commercial production of tissue-engineered skin products

Commercial production of tissue-engineered skin products takes place in a precisely controlled and highly regulated environment, which imposes many constraints if the process is to be successful. These will be discussed under a series of headings, dealing with physical constraints, such as bioreactor design, growth system design, cell sources, and with broader considerations of the nature of preclinical and clinical assessment, government regulation, reimbursement, and the market place. It is central to a commercial enterprise that it ultimately make a profit if it is to be successful. Since tissue engineering is inherently an expensive undertaking, minimizing costs at all stages is essential.

Regulation

Regulation of tissue-engineered products by the Food and Drug Administration (FDA) or other regulatory authorities has taken a long time to develop. Since commercial products have only been available for less than 20 years and they are very different from other medical products, establishing a regulatory pathway has been complex. Tissue-engineered skin substitutes do not fit readily into any of the standard FDA categories; for drugs, the Center for Drug Evaluation and Research (CDER); for biologics, the Center for Biologics Evaluation and Research (CBER); or for devices, the Center for Devices and Radiological Health (CDRH). Classification is important as requirements, criteria and guidelines differ substantially between the centers. Skin implants were originally considered as dermal or skin replacements and classified as devices. As it became evident that growth factor activity played a part in their mode of action, considerations appropriate to biologics played an increasing role. There is now within FDA an Office of Combination Products responsible for assembling reviewers from different FDA centers to form a team to review such products. While tissue-engineered skin substitutes generally have more biochemical activity than would be expected of a pure device, they do not fit easily as a biologic. For instance, the concept of "dose" is not straightforward. If a component of the activity of these materials is through secretion of growth factors, growth factor production would seem to be a reasonable release criterion. However, unlike a purified growth factor preparation, these products are living, and the secretion of growth factors may vary depending on conditions. The cells adjust their cytokine output to the environment in the wound bed. It may be possible to devise conditions under which secretion of a growth factor is maximal and determine output under those conditions. For instance, vascular endothelial growth factor (VEGF) might be measured under optimal PDGF stimulation. However, this has not been explored and to avoid such problems, a "dose" of Dermagraft has been defined as a piece.

Also, at this stage, the composition of tissue-engineered skin can only be determined to a limited extent. Usually, it is desirable that a biologic contains well-defined constituents, including an active ingredient at a known dose and excipients. Thus far, studied components of skin implants have included those that are known, and, to some degree understood, from other work. There is no comprehensive identification of constituents, and several that appear on expression arrays, such as lumican and collagen type 6, which are prominent, have received inadequate attention.

However, despite these difficulties, the FDA is placing increasing emphasis on the biological aspects of skin substitutes and overseeing them as biologics and as drugs.

Product development

Development of a commercial product should start with a product concept from which a design requirements document is developed. As an example, Table 79.2 shows the product concepts for Dermagraft and TransCyte. The product is then developed to meet the design requirements by a series of hierarchical, more and more detailed design processes that ultimately comprise the design master file. The requirements can be divided into groups: in each concept, the first statement is concerned with the therapeutic purpose of the product, the next three describe intrinsic characteristics of the product, and the last three are concerned with practical issues of importance to the final user. All these factors need to be considered from the earliest stages of development.

Overall concept

The initial application for Dermagraft was a dermal replacement for burn patients. Initial clinical application indicated that it provided little benefit and was slightly

TABLE 79.2 Design concepts for Dermagraft and TransCyte.

Dermagraft	TransCyte
Dermal replacement, later as a cytokine factory	Transitional covering for third-degree burns to replace cadaveric skin
Allogeneic	Allogeneic
Viable product	Nonviable
No sterile fill, that is, bioreactor would be package	No sterile fill, that is, bioreactor would be package
Long shelf life (~6 months)	Long shelf life
Suitable size	Suitable size (from Biobrane size)
User-friendly presentation—modified bag	User-friendly presentation—clamshell bioreactor

deleterious. As trials for chronic wound applications progressed, and it became evident that it was efficacious, the concept of Dermagraft as a cytokine source replaced the notion of a dermal replacement.

TransCyte, a replacement for cadaveric skin for covering third-degree burns after debridement, was seen as a means to overcome problems with rejection and potentially with disease transmission.

Allogeneic cell source

The second item in both product concepts was that the products would be allogeneic. The decision whether a product will be allogeneic or autologous is fundamental to the entire development process. While not impossible, the development of a commercially successful (profitable), autologous, tissue-engineered product is very difficult. It requires separate, independent, tissue culture suites (hood, incubator, centrifuge, microscope, and air conditioning), which will be occupied by the product for a single individual for the entire period of culture. It is a service industry, with complex logistics and high cost. As an alternative, it would be possible, in principle, to construct an automatic machine that would accept a patient's tissue and produce the tissue-engineered construct in the hospital with minimal intervention. The advantages of an autologous process from a commercial point of view is the comparatively low requirement for safety testing and the lack of immunological rejection by the patient.

Allogeneic tissue engineering has advantages in using only one tissue culture facility per product type, allowing economies of scale and straightforward logistics. The major disadvantages are the very large amount of safety testing required for the master cell banks (MCBs) and the potential for immunological rejection. The first of these disadvantages is ameliorated by the infrequency of making MCBs. In the case of Dermagraft and TransCyte, a single MCB was used for more than 20 years, so the investment could be amortized over a long period. Required testing is determined by regulatory authorities and may be increased from time to time as new risks become known. It includes testing of the donor and, in the case of fibroblasts, the donor's mother for major human pathogens (human immunodeficiency virus, hepatitis, cytomegalovirus, etc.). The cells isolated from the donor are tested for the major human pathogens, xenogeneic pathogens (which might be introduced from the reagents used in tissue culture), karyotype, identity (isoenzyme, variable number of tandem repeats, single nucleotide polymorphisms, etc.), tumorigenicity, latent viruses, etc. This is tested both on the initial MCB and again on cells reisolated from final product (the end-of-production cell bank).

Viability of product and avoidance of a final sterile fill

The viability of the product depends on its intended function and differs for Dermagraft and TransCyte. As discussed later, to package sterile material is a matter of some difficulty and it was decided, in this case, to avoid the issue. The bioreactor in which the tissue was grown was designed to form part of the final packaging.

Shelf life

Extended stasis preservation is important for commercial products, including tissue-engineered products. It allows time for quality control (QC) testing, for off-the-shelf availability, and for distribution. In addition, the Dermagraft and TransCyte processes take 12 weeks from thawing a vial from the manufacturer's working cell bank (MWCB), cell expansion, 3D growth, and QC testing. The difficulty of predicting the market so far ahead so as to minimize waste is greatly aided by extended shelf life.

This was achieved through cryopreservation at −70°C. Other systems for long-term stabilization of tissue-engineered constructs have been explored with varying degrees of success, including trehalose, DMSO, and 3-*O*-methyl glucose [27−29].

Size, user convenience

The remaining items in the product concept are related to convenience of use by the physician applying the product. This is an important item for a commercial product and needs much work in focus groups and thought. Thawing the product is already a major issue, requiring time and, in the case of TransCyte, careful estimation of the amount required to avoid wasting expensive material or having to wait for a piece to be thawed. The Dermagraft bioreactor was designed so that it would open easily and the tissue would be positioned so as to be easily rinsed prior to use, through the use of a *Z*-weld that attached opposite sides of the scaffold to opposite walls of the bioreactor. The bioreactor is translucent to aid in marking out the piece to be cut out for implantation.

The manufacture of Dermagraft and TransCyte

Overall schemata for the manufacture of Dermagraft and TransCyte are illustrated in Fig. 79.1. As can be seen, as much of the two processes as possible are identical. This economizes resources and reduces cost. In this case the MCB, MWCB, the cell expansion process, and much of the release testing are all in common. In addition, the clean room facility, all its ancillary equipment and monitoring are the same.

Cells

Several times per week, fibroblasts used in manufacture are recovered from liquid nitrogen storage (the MWCB) and expanded in roller bottles from fifth to eighth passage. This is a conventional procedure but may not be ideal. Fibroblasts for Apligraf are expanded on beads in a mixed bioreactor. It has become evident that the proliferation of fibroblasts is improved at low oxygen (2%−6%) [30], and, at this oxygen concentration, the development of a senescent phenotype may be reduced [31−33]. A tank bioreactor lends itself to active control of parameters such as oxygen tension and pH and is worth exploring before a system gets fixed by regulation. The cells should ideally be grown just to the beginning of stationary phase. Further incubation seems to be slightly deleterious.

Expansion of fibroblasts for Dermagraft and TransCyte manufacture was performed in roller bottles. These are well established for the production of large numbers of cells under ambient conditions. Various other systems have been devised that may provide superior performance and greater control of the conditions of growth [34,35].

Medium

The Dermagraft and TransCyte systems are grown in Dulbecco's-modified Eagle medium, supplemented with 10% calf serum, nonessential amino acids, and glutamine. All tissue culture beyond initial cell isolation from the foreskin is free of added antimicrobial agents, such as antibiotics. Antimicrobial agents might hide otherwise significant contamination, which might only become evident in therapeutic application.

With the increased concern with the transmission of prion diseases, there has been great interest in developing serum-free systems. While serum-free media are available

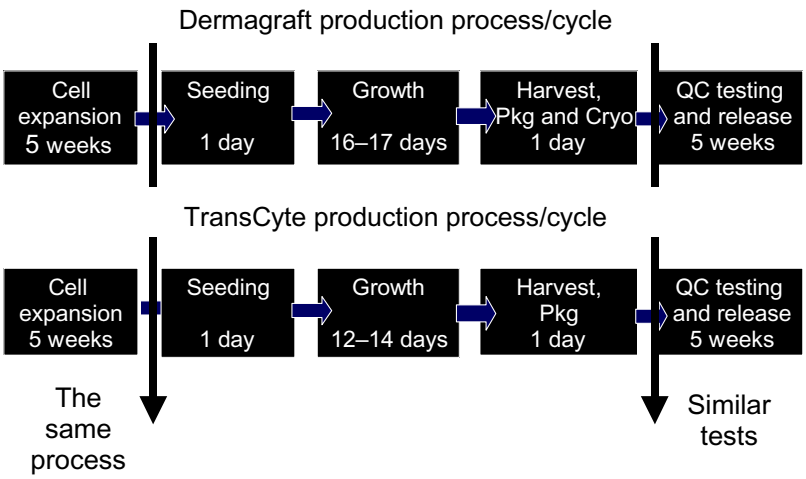

FIGURE 79.1 Overall comparison of the Dermagraft and TransCyte processes.

for keratinocytes, it has been found difficult to achieve such systems for fibroblasts. Serum-free fibroblasts media are commercially available, but while these have been successful in the research laboratory, transferring them to scaled-up systems and manufacturing has proved elusive. The major issue is that the cells do not grow to the extent required apparently because they acquire a senescent phenotype comparatively early in the expansion process. It has long been established that serial subculture of fibroblasts leads to senescence after about 60−80 population doublings. While this can be attained in serum-containing medium, experiences with serum-free media have indicated a life span of about nine doublings. Since Dermagraft is made at about the 28th generation, which is about 10 doublings from the MWCB, 19 from the MCB, and because implanted fibroblasts should not be too close to senescence, serum-free media do not provide sufficient proliferation potential.

The alternative solution to serum-free medium is to take extreme caution in the source and treatment of serum. By regulation, all materials used in manufacture must be traceable to their sources, but in addition, bovine serum is obtained only from countries that are free from bovine spongiform encephalopathy, which include Australia and New Zealand, from closed herds with a known pedigree history that includes no animals from outside the country. Serum is also irradiated sufficiently to eliminate possible viral contamination.

Bioreactor design

Many of the considerations of bioreactor design involve questions of mass transport, conditions under which cells are able to grow and so forth, much as in noncommercial tissue engineering systems. A second series of considerations deals with the large-scale production process. These include ease of scale-up, ease of handling, minimal footprint, and maximal automation. In addition, the use of the product by a community not experienced with products of this type is also important. This leads to an emphasis on features to make the product easy for the end user to work with and apply.

The bioreactor used for the growth of Dermagraft consists of a bag with eight cavities welded into it, each of which contains a sheet of the scaffold. Such bags are attached in groups of 12 to manifolds, with a solid support, for cell, medium or cryopreservative addition, to form a system. Several systems may be connected and all fed at the same time to form a lot, which is the unit of manufacture. The bag reactors, systems, and even lots may be assembled under clean, but not necessarily sterile conditions and sterilized by 25−43 Grays gamma radiation prior to seeding. From this point the process only requires nine sterile connections and is substantially automatic.

The TransCyte bioreactor systems are similarly assembled under clean conditions, complete with all associated tubing, and attached in groups of 12 to a manifold to form a system. This entire assembly is then sterilized by gamma radiation as described previously. Several systems can then be connected to form a lot. In this case the inlets to each bioreactor are individually passed through a peristaltic pump to provide even flow. (Attempts to use constrictions to control flow were found to be inferior.) Again, as with the Dermagraft system, once set up and seeded, the TransCyte system is substantially automatic.

Automation in tissue engineering systems is an important feature. Apart from reducing labor costs, automation greatly reduces the number of errors that have to be investigated, requiring time, effort, and cost. While operator errors are generally less serious than machine failures, they are much more numerous and still require individual investigation. Backup systems can be installed to minimize the impact of machine failure.

The Dermagraft and TransCyte production processes

The production of Dermagraft and TransCyte started with the cryopreserved MCB that was stored at passage three in liquid nitrogen. About four times per year, cells were taken from this bank and expanded in roller bottles to fifth passage when they were stored again as the MWCB. This was tested to ensure identity to the MCB. Several times per week, cells were taken from the MWCB and expanded to eighth passage in roller bottles when they were seeded to the 3D bioreactors. This process was conventional, large-scale tissue culture. It used batch feeding and exposed the cells frequently to atmospheric oxygen. As discussed elsewhere in this article, fibroblasts grow better and senesce less rapidly in a lower oxygen environment.

On seeding, the bioreactors were manipulated to ensure even distribution of the cells. In the case of Dermagraft, the bioreactors were rolled; in the case of TransCyte, each side was seeded successively under static conditions. The medium was replaced 1 day after seeding and then the cells fed every 2−4 days until harvest.

The time of harvest was determined from the glucose metabolism of the cultures. In the case of TransCyte, glucose consumption and lactate production were determined daily and interpreted as notional adenosine triphosphate (ATP) turnover, assuming a P:O ratio for mitochondrial oxidative phosphorylation of 3. The resulting values were correlated with the performance of the system relative to release specifications and appropriate target values

selected. In the case of Dermagraft, there was a concern that lactate release from the degradation of the scaffold might interfere with the ATP turnover estimation, so the time of harvest was determined on cumulative glucose utilization alone.

At the harvest of Dermagraft, medium was replaced by a cryopreservative solution that consisted of 10% dimethylsulfoxide in phosphate buffered saline, supplemented with calf serum, and the individual pieces of Dermagraft sealed, removed from the surrounding bag, packaged, cooled slowly at a rate that fell within design parameters, and frozen. It was then stored in a freezer set to −75°C. The time between cryopreservative addition was at least 4 hours and was validated to a 12 hours delay. This time interval did not appear to affect the viability of the cells and it was conjectured that it allowed induction and synthesis of stress proteins in response to the high osmotic pressure.

Release specifications

The release specifications included sterility (bacterial, fungal, mycoplasma, and endotoxin) as well as analytical criteria that ensure consistency of the product. Dermagraft used four analytical release criteria, 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) reductase activity before freezing (in-process MTT) and after thawing, collagen content by Direct Red binding [36] and DNA content. MTT reductase was used to determine the metabolic activity of the product, collagen as a surrogate for the amount of extracellular matrix, and DNA content was used to establish adequate cellular content. MTT reduction was an assay that was established early in the development of Dermagraft. Since then, several other substrates measuring essentially the same activity have been developed, several of which are less toxic to the cells than MTT and can be used repeatedly. It would be wise in the development of a new product to examine these early and select a method carefully. MTT reductase is frequently viewed as measuring mitochondrial activity. However, 70% of the activity is extramitochondrial [37] and it is better regarded as a general measure of cellular activity. The MTT reductase assay was originally developed to evaluate toxicity or cell proliferation, where values obtained after treatment were compared with pretreatment control values. In determining the metabolic activity of tissue-engineered products, this is not possible and a great deal of fruitless effort has been expended in trying to obtain a standard for the MTT assay. MTT determination before and after cryopreservation might be interpreted in the same manner as in toxicity assays. However, changes occur in the viability and metabolic activity of cells for up to 3 days after cryopreservation, due to necrosis, apoptosis, and repair; so the MTT values for tissue-engineered products are better regarded as independent absolute determinations rather than relative values.

In the case of TransCyte the microbiological criteria and the collagen and DNA determinations are similar to those for Dermagraft. However, as the cells are nonviable in the final TransCyte product, there is no MTT determination. Determination of MTT reductase prior to final freezing (in-process MTT) was replaced by release specifications applied to ATP consumption as described earlier.

Definition of specifications was initially within three standard deviations of the process performance. In the case of MTT, it was evident during the clinical trial that the specifications needed to be refined, requiring a mid-trial correction and ultimately an additional clinical trial.

Distribution and cryopreservation

The use of −70°C cryostorage for Dermagraft was a compromise between an ideal solution and practicality and turned out to have several benefits. If the goal was 100% cell viability and indefinite shelf life, the ideal solution was likely to have been storage at liquid nitrogen temperatures, probably using a vitrification procedure in which high concentrations of cryoprotectant and very rapid cooling is used to achieve the formation of a glass with minimal ice crystallization. While the technical difficulties of achieving sufficiently rapid cooling of a therapeutically useful structure and problems with the brittleness of plastics at low temperatures can probably be overcome, the method would still entail transport at liquid nitrogen temperatures and safety issues. Cryopreservation at dry ice temperatures provided adequate shelf life and allowed shipping in a container capable of maintaining −65°C for 4 days. This allowed distribution throughout the world and would generally permit 2−3 days leeway for patient and physician scheduling at the site of use. If a center had a −70°C freezer, storage at this temperature would allow much longer shelf life at the treatment site.

In addition to logistical advantages, the induction of stress proteins by the cryopreservation procedure [38], combined with the suboptimal viability recovery meant that necrotic cell debris, including heat shock proteins, was released from lysed cells. Such proteins activate macrophages through CD91 and Toll-like receptors [39]. While such necrotic products are likely not to be scarce in a chronic wound, this property may have contributed to the efficacy of Dermagraft.

While discussing cryopreservation, an important point that has received little attention is the thawing process. In the case of Apligraf the thawing protocol involved multiple solution replacements, requiring a procedure of several hours, which was performed at the company. This provided shelf life, but not off-the-shelf convenience, and

required additional resources from the company. Therefore the procedure was not continued. In the case of Dermagraft, thawing was performed in the physician's office, out of the control of the company.

During the controlled freezing process, after the majority of the osmotic water removal has occurred, the cell contents reach a eutectic and freeze. While most of the intracellular material forms a glassy state, small ice nuclei may be formed. The slow growth of these nuclei during −70°C storage is probably what limited the shelf life of Dermagraft.

During thawing, conditions arise that allow the growth of ice crystals, which enlarge and, if they become big enough, kill the cells. It is important to take the cells through this stage as rapidly as possible to minimize ice crystal growth. So rapid thawing, straight from the −70°C storage is critical. The procedure that was adopted for Dermagraft involved a thawing tub with a thermometer, which could be filled with warm water from the tap, or a water bath at 37°C. A few seconds of exposure of frozen Dermagraft unprotected in ambient air was sufficient to reduce viability drastically. It may be noted here that the viability of Dermagraft could probably been improved be decreasing the amount of cryoprotectant in the final product, reducing its heat requirement, and thus allowing more rapid thawing.

Problems with commercial culture for tissue engineering

In large-scale tissue culture, procedures are followed that differ from those found in a research laboratory. As a result, problems have arisen from time to time that have no counterpart in culture on a smaller scale. Frequently, it is difficult to establish the cause unequivocally because of the scale and cost of the experiments that would be required. When such a problem occurs, it is usual to assemble all those with potentially valuable ideas, analyze the incident with respect to changes in procedure (new batches of medium or other raw materials, newly implemented changes, etc.), and prioritize possible solutions. Usually the most likely and cheapest suggestions are explored first. These tend to be implemented more or less at the same time and immediately. This means that, when the problem ceases, as it usually does, it is very difficult to determine which suggestion solved it. At best, the solution relies on a plausible rationale.

Examples of the causes of such occurrences include light damage to medium and deterioration of cell banks. It has been known for many years that medium is light sensitive and will degrade if left exposed to daylight or fluorescent light. However, such an event is never seen in the research laboratory, as medium is normally stored in a refrigerator in the dark and exposed to light for short periods. In commercial operation, it is sometimes necessary to make up medium a few weeks in advance and store it in a cold room. If the lights in the cold room are fluorescent and are left on, deterioration of medium will be observed. Indeed, the Dermagraft plant was illuminated with yellow light to avoid such problems.

In a research laboratory, cell stocks frozen in liquid nitrogen are manipulated rarely, a few times per year. In commercial-scale tissue engineering, vials from the MWCB are required several times per week. While one or two vials are identified and removed, the remainder of the cell bank stock taken out of cryostorage is warming up. We noticed that, after some years of this, the fibroblasts could be shown to demonstrate a decreased life span. This was attributed to ice recrystallization during periodic warming to perhaps −120°C to −90°C during recovery of other vials. It is, thus, necessary to store the MCB independently of the MWCB and to devise a method of recovering selected vials with minimal disturbance to other vials.

Clinical trials

The design and implementation of clinical trials is specialized and beyond the scope of this discussion. The process requires a great deal of time and expense. In the case of the CDRH division of the FDA, the initial trial is a pilot or feasibility trail, equivalent to a Phase I trial in the CBER or CDER. This consists of a small number of patients (6−20) per treatment regime and is primarily concerned with safety. Any information obtained on efficacy in a trail of this magnitude is unlikely to be statistically significant but may provide useful information on the size and variability of the therapeutic effect that is valuable in design of the subsequent, pivotal trial.

The pivotal trials (equivalent to Phase III trials in biologics and devices) involve much larger numbers of patients (50−500 in the case of devices), which is determined by a sample size calculation, based on the results from the pilot trial, to give about 80% probability of a significant result ($P < .05$) if one exists. Recently, it has been suggested that the threshold value of P should be reduced to .005 to reduce the number of false successes, which can lead to much further development expense before the product definitively fails.

Trials with chronic wounds take about 2 years or more to complete. The design phase, deciding on protocols, obtaining institutional approval for multiple centers and preliminaries to the recruitment of patients is likely to take at least 6 months. The actual trial involves screening many more patients than will actually take part in the trial, as many fail to conform to exclusion criteria, which reduce irrelevant and interfering factors, established in the

design phase. With a trial involving a 12-week follow-up period, most should be completed within 18 months to 2 years. There is then a period of 6 months while the data are checked, discrepancies resolved and the results of the trial evaluated. This period could be shortened substantially by logging the patient outcomes to an internet site and checking them online as they are generated.

The design of the clinical trial should be conducted in close consultation with the relevant regulatory authorities. It is critically important that all aspects should be thoroughly discussed and agreed beforehand. In the case of Dermagraft, failure to follow advice from the FDA precisely led to considerable delay and additional expense.

Considerations in the selection of clinical trials include the size and value of the potential market, the expected efficacy of the product, and the difficulty of completing the trials. Five major areas of chronic wounds include venous stasis ulcers, diabetic foot ulcers, arterial ulcers, pressure (decubitus) ulcers, and all other chronic wounds. Of these, venous stasis ulcers represent the largest market. Diabetic ulcers are a valuable market, as, while smaller in number, the consequence of failure to heal such an ulcer is limb amputation, an expensive and seriously debilitating procedure. It is very difficult to perform a trial on decubitus ulcers, as the patients are frequently elderly, infirm and may need approval from a guardian. Such trials are very arduous, may be difficult to complete and are best undertaken by a specialized group with well-developed access to such patients. In no cases does the treatment of chronic wounds with skin implants address the underlying cause of the ulcer, which lies in venous insufficiency, metabolic abnormalities, reduced arterial supply, or repeated ischemia. This is particularly evident in ulcers caused by arterial insufficiency. While the angiogenic activity implant may be capable of improving vascularity in the region of the wound, little may be gained if blood supply to the limb is inadequate.

The pivotal clinical trial for Dermagraft for diabetic ulcers was initiated in 1994. Dermagraft gave a statistically significant increase in healing of 19.1% that was similar to results obtained in similar trials with other products. These data were used in combination with mechanism of action and clinical instruction to support the clinical use of Dermagraft commercially. Since then, it has grown to a several $100 M/year business.

The indications for which the product may be marketed (its labeling) are strictly limited by the clinical trials and new trials are required to extend them. However, in certain categories, there may be insufficient patients to support both a clinical trial and a commercial market. Examples are monogenetic congenital diseases, where, although the conditions may be very distressing, the entire population may number in the hundreds or a few thousand. An example in the chronic wound field is epidermolysis bullosa (EB). For such diseases the FDA has a category, humanitarian device exemption (HDE), which allows much reduced clinical testing. It does, however, require institutional review board approval from each institution where it will be used. In the case of dermal implants, such a route has been used for Apligraf and Orcell for all genetic forms of EB, and for Dermagraft for the dystrophic forms of EB. The category, "all other chronic wounds" includes many miscellaneous conditions, such as pyoderma gangrenosum and necrobiosis lipoidica diabeticorum, for which there is too small a patient population to perform a trial, but it would be expected to benefit from treatment with Dermagraft or TransCyte. The HDE pathway may not be appropriate or available. However, since the FDA does not regulate the practice of medicine, some approach to these patients may be obtained through off-label use, although this cannot be formally promoted by the manufacturer.

Immunological properties of tissue-engineered skin

Immunological rejection of tissue-engineered products remains a controversial question. As was clearly established by Medawar in the 1940s, allogeneic transplants of whole live organs invariably cause immunological reactions that lead to rejection [40]. However, experience with some cultured cells and tissue-engineered constructs indicates that, in some cases, no clinically significant rejection occurs. At this point, some hundreds of thousands of patients have been implanted with Dermagraft, in many cases several times to the same patient, without a single example of immunological rejection and experience with Apligraf has been similar. The fibroblasts from Dermagraft have been found to persist in the wound site for 6 months and survival for about a month has been obtained with Apligraf [41].

There are two major pathways of transplant rejection, direct and indirect. The direct pathway involves recognition of donor histocompatability antigens (HLA) by the host immune system leading to acute rejection over about 2 weeks. This involves both HLA, of which Class II molecules, such as HLA-DR, are the most important and also costimulatory molecules on the transplant, CD40 and the CD80 group. These molecules are not normally expressed by fibroblasts. However, in monolayer tissue culture, the presence of γ-interferon, which may be present in chronic wounds, will cause induction of HLA-DR, CD-40 and genes involved with the physiological function of Class II HLA (antigen presentation). In contrast, in 3D culture, many of the cells show a selective response to γ-interferon, which excludes induction of these molecules [42]. The indirect pathway of rejection involves display

of transplant antigenic peptides by host antigen presenting cells (macrophages, tissue dendritic cells, and endothelial cells). This gives rise to chronic rejection which may cause destruction of the transplant over many months. While it cannot be excluded that this may occur, Dermagraft fibroblasts have been detected at 6 months from the time of implantation, and no clinical evidence for chronic rejection has been observed.

Acute rejection is primarily an attack on the endothelial cells of the vascular system, which are antigen presenting cells and do express the component of the antigen presentation system (HLA Class II, CD40, CD80). It is possible that the lack of rejection of tissue-engineered skin products is related to the absence of such cells. Indeed, adding antigen presenting cells (in this case, B lymphocytes) to a tissue-engineered construct has restored susceptibility to immunological rejection [43].

Commercial success

There are many contributors to the commercial success of a product. They include a satisfactory clinical trial, a good rationale for the performance of the product, convenience in use, a reasonable price, adequate reimbursement from third-party payers, sensitivity to complaints, and efficient distribution. Some of these require long-term effort and must be initiated early in the development process.

New products frequently find themselves selling into a conservative and skeptical market that arises from the hype that is involved in developing and funding the product. The first requirement in overcoming this view is a satisfactory clinical trial. This process is determined by regulatory bodies. The major criteria for the American authorities (FDA) are safety and efficacy. Unless the product's safety and efficacy is extraordinary, clinical results need to be backed up by a mechanism of action that, while it may not be fully established, must provide a logical reason for using the product. In the case of Dermagraft, this has led to a great deal of fundamental wound healing research.

Equally important is the convenience of use, cost, and process for reimbursement of the physician applying it. Convenience of use and cost are functions of bioreactor and process design, and company structure, which are discussed elsewhere. Third-party reimbursement and health economics are major and specialized subjects that are outside the scope of this article. However, a major component of establishing satisfactory reimbursement in the United States involves the Center for Medicare and Medicaid Services (CMS). Government authorities with similar responsibilities are involved in other countries. Their criteria, in contrast to the FDA, are reasonableness and effectiveness/cost ratio. Both the FDA-type and CMS-type criteria have to be met for a product to be successful.

Following introduction into the market, products approved under CDER are tracked. Adverse events, new toxicities, and complaints that might affect the use of the product have to be reported. However, performance has not been. It may, however, become a regulatory requirement to confirm safety, efficacy, and cost-effectiveness with a larger patient population than possible in a clinical trial. It is also valuable to the manufacturer because, while anecdotes of positive clinical experience may be heartening, they do not provide objective quantitative data. This was not performed in the cases of Dermagraft or TransCyte, making it difficult to assess the actual performance of the product in clinical use.

Mechanism of action

Understanding the mechanism of action of a product required some understanding of the underlying pathology of the disease the product is intended to treat. Wound healing, and particularly the etiology and maintenance of chronic wounds, was poorly understood during the development of Dermagraft and TransCyte. Many phenomena have been observed, particularly, in chronic wounds. The problem is sorting out how they relate to one another, which are etiological, and which are symptomatic; the difference between cause and response. The problem was less acute in the case of TransCyte as the product was only designed as a temporary covering for third-degree burns.

Changes from normal skin observed in chronic wounds include lack of keratinocyte migration, capillary cuffing, protease activity, abnormalities in macrophage activation, diminished vascular supply, and senescence in fibroblasts [44]. In developing a rationale for the action of Dermagraft, the first aspects considered were vascular supply and keratinocyte migration. Much study was undertaken on the angiogenic activities of Dermagraft and it was shown that the tissue produced VEGF and hepatocyte growth factor. It was also shown to be angiogenic in the chick chorioallantoic membrane assay and to increase blood supply to diabetic ulcers in patients in vivo using laser-Doppler techniques. Thus a case could be made that one mode of action was through its angiogenic effect.

The high protease activity of chronic wounds was combined with the lack of keratinocyte migration to hypothesize that the extracellular matrix in a chronic wound was degraded to the extent that the migration substrate was lacking. Indeed it was demonstrated that fibronectin was degraded by neutrophil elastase [45]. This led to work on the properties of Dermagraft and TransCyte as substrates for keratinocyte migration.

During comprehensive surveys of genes induced by 3D culture, it was found that the most highly upregulated gene was IL-8. Other ELR CXC chemokines were also highly induced. This led to studies of the secretion of these proteins and to changes in their production that might relate to the etiology of chronic wounds and the possible role of Dermagraft. It was found that Dermagraft secretes IL-8, sometimes in large quantities although the secretion was extremely variable. Monolayer fibroblasts also secrete CXCL-1, CXCL-5, and CXCL-6 that are all chemoattractive for neutrophils and activate them to a bactericidal phenotype. At the same time, it was observed that bacterial products (lipopolysaccharide) inhibit keratinocyte migration. This led to the hypothesis that a major function of fibroblasts in a wound context is the recruitment of neutrophil leukocytes to destroy colonizing bacteria and that failure of this system in chronic wounds results in the establishment of bacterial contamination that leads to failure of reepithelialization. It is known that fibroblasts in chronic wounds display a senescence-like phenotype [46] that grows slowly and is unresponsive to growth factors. The condition is probably related to stress-induced premature senescence caused by ischemia reperfusion injury, metabolic abnormality, extravasated red cells, and the inflammatory conditions of the chronic wound. It has been found that, although the senescent fibroblast phenotype is generally inflammatory, the production of CXCL-1, CXCL-5, CXCL-6, and CXCL-8 decline in senescent fibroblasts. Thus in chronic wounds, decline in the ability of the fibroblasts to recruit and activate neutrophils allows wound colonization by bacteria and failure of keratinocyte migration to close the wound. Chronic wounds seem to involve an arrest of normal healing at about the stage of neutrophil immigration. On this hypothesis a major role of Dermagraft is to provide nonsenescent fibroblasts that are able to respond to the presence of bacteria with appropriate secretion of neutrophil chemoattractant chemokines.

In the case of TransCyte the formation of a foreign body capsule–like material in vitro leads to lack of ingrowth of the scaffold into the wound through a host foreign body reaction, and, thus, comparative ease of removal. The ability of TransCyte to reduce the pain of second-degree burns is common to many occlusive dressings.

A remarkable feature of dermal implants is that they do not appear to have led to an increase in infection. Indeed the general experience has been a slight reduction in infections. Preparations, including keratinocytes, show secretion of antimicrobial peptides, but this is not known to be the case for fibroblasts. The most likely explanation is a combination of fibroblasts secretion of neutrophil chemoattractant CXC chemokines and the provision of an extracellular matrix substrate for leukocyte migration.

Future developments

The major direction of development in skin products is toward simpler, possibly nonviable systems. Inclusion of live cells in a product entails many issues such as the use of allogeneic cells, cryopreservation, distribution, inconvenience to the customer (for instance, the thawing procedure), and many manufacturing problems. Hence, studies were initiated to explore the possibility that a possibly less effective but substantially less expensive product could be developed. This is a direction that has also been explored by companies in the field.

It is a general principle that value is likely to be obtained by exploiting a technology base as far as possible. A major product of fibroblasts is collagen, in this case human. As a nonviable product, human collagen has been extracted from the extracellular matrix laid down by fibroblasts in 3D culture and used, by injection, for the treatment of wrinkles.

An alternative to cryopreservation that has been explored is the possibility of storage by desiccation. Many natural systems, such as plant seeds, tardigrades, *Artemia*, and yeast have developed the ability to survive desiccation and, in that state may be able to survive extreme conditions of temperature, vacuum, radiation, and time [47]. Many factors appear to be involved in this ability [48], but one that has attracted attention is trehalose, which is a disaccharide (1,1,α,α diglucose) that is produced in large quantities by many desiccation-resistant organisms. It was found that TransCyte could be dried at room temperature in the presence of trehalose, irradiated to 25–40 Grays to provide terminal sterilization and would recover its structure and wound adhesion on rehydration. Similar results could be obtained by careful lyophilization. While some success has been obtained in the retention of viability of human cells in the desiccated state, it is not yet possible to obtain the shelf life (at least 1 month and preferably at least 6 months) required for a tissue engineering application. It is also questionable whether the radiation treatment possible with a nonviable product would be appropriate with living cells. In this case an aseptic drying and packaging system would have to be devised.

Thus far, the therapeutic applications of skin tissue engineering have encompassed acute and chronic wounds and comprise dermal components alone or dermal components with epidermal structures. In the future, it is likely that adnexal structures will be added. Remarkable success in this direction has been achieved with hair follicles [49], where it has been found that, in a mouse system, hair follicle-inducing cells can be cultured so that they retain their properties and, mixed with hair follicle-derived keratinocytes, will on injection form a hair. Similar results have also been achieved with human cells. The

remarkable feature of this system is that the entire organ, hair follicle, sebaceous gland, and erector pili muscle is formed. This constitutes one of the few examples of true organogenesis observed using adult cells. We may also expect the increased use of induced stem cells.

Conclusion

Experience with the commercial production of tissue-engineered skin has developed many principles that are important beyond the simple development of a tissue-engineered product. Most of these are based on well-known concepts used in other manufacturing processes, as applied to tissue engineering. They include

1. optimized cryostorage of MCBs with minimal access
2. intermediate storage of expanded cell banks (MWCB)
3. use of allogeneic cells
4. bioreactors that permit scale-up and minimize footprint
5. minimal aseptic connections
6. maximal automation, to minimize errors
7. avoidance of aseptic fill, so that the bioreactor forms part of the final package
8. bioreactor designed for end user convenience
9. cryostorage

While it may not be possible to incorporate all of these principles into the manufacture of a tissue-engineered product, as many as possible should be used. The major problem with such products in the market place is cost, and all these principles are directed toward reducing cost. Some, such as the use of allogeneic cells, may be difficult to implement. While experience with fibroblasts indicates that immunological rejection is not a major issue, this is not known for other cells. The possible use of allogeneic cells should, however, be checked, preferably in animals other than mice, because the advantages for a commercial product are great. Optimal cryostorage is a compromise between ideal tissue survival and other factors, such as ease of distribution.

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Chapter 80

Tissue-engineered cartilage products

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Introduction

The articular cartilage provides an extremely smooth surface, which significantly reduce the friction between the ends of articulating bones. Individual diseases affecting the cartilage include traumatic chondral defects, osteochondritis dissecans (OCD), osteonecrosis (ON), and osteoarthritis (OA), which are profoundly different in their origin. Articular cartilage defects caused by trauma, OCD, and ON are potentially severe pathologies because they may induce (secondary) OA. Of importance, OA is a heterogeneous disorder, caused by special biomechanical, genetic, metabolic, and inflammatory events affecting all structures of the joint, including the articular cartilage, subchondral bone, menisci, and synovial membrane. Thus the usually ill-defined large OA cartilage lesions should not be confused with the often well demarcated, circumscribed chondral defects, as only the latter are amenable to regenerative cartilage repair techniques.

After early preclinical experiments—such as transplantation of autologous chondrocytes isolated from nasal septal cartilage of rabbits into muscle, where new cartilage is formed [1], and transplantation of autologous epiphyseal and articular chondrocytes into damaged joint surfaces of rabbits [2]—and studies in the laboratory of Daniel Grande providing evidence that the repair tissue in cartilage defects is based on the transplanted cells using autoradiography [3], autologous chondrocyte implantation (ACI) was first performed by Lars Peterson in patients in 1987 and published together with Mats Brittberg in 1994 as the first clinical tissue engineering application. ACI is a two-stage surgical procedure characterized by a first step of initial (arthroscopic) cartilage removal, cell isolation, and expansion, and the second step of subsequent implantation. While the initial technique of transplantation involved injecting a cell suspension under an autologous periosteal flap retrieved from the ipsilateral proximal tibia, it has seen significant advances that have further strengthened the clinical process of ACI through modern biotechnological innovations, for example, by applying biomaterials as cell carriers. However, the basic principles of ACI continue to involve the removal of cartilage, chondrocyte isolation, and expansion in the laboratory, and the subsequent reimplantation into the cartilage lesion. The goal of ACI, as for all surgical procedures, is to achieve a high degree of articular cartilage repair. In this sense, ACI continues to reflect the very principles of tissue engineering, as defined by Robert Langer and Joseph Vacanti in 1993 as "an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function."

Supported by the technical advances, ACI has clinically and scientifically established itself in recent years. For example, there are currently long-term results with follow-up periods up to 20 years after implantation, and the number of high-quality controlled prospective randomized studies has increased significantly, also against the background of the regulatory requirements, resulting in an increased scientific proof of the effectiveness of the method. Also, the recent cost-effectiveness assessment by the National Institute for Health and Care Excellence (NICE) in Great Britain concluded to strongly recommend ACI as an option for treating symptomatic articular cartilage defects of the knee for defect sizes above 2 cm^2.

Cartilage defects, osteoarthritis, and reconstructive surgical options

Cartilage defects pathophysiology

By definition, cartilage defects involve a disturbance of the structural continuity of the articular cartilage. Chondral defects are restricted to the articular cartilage and are classified either as partial- or full-thickness

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chondral defects based on their depth. Osteochondral defects also disrupt the structural integrity of the subchondral bone, representing an extension of the purely chondral defects, for example, as a result of an osteochondral fracture. Chondral defects are only sparsely repopulated by cells which are migrating from the synovial membrane. The spontaneous repair of osteochondral defects is much more efficient, because mesenchymal stem cells (MSCs) from the bone marrow (BM) compartment (BM-MSCs) initiate a significant reparative response. There are several factors affecting repair, among which patient age, defect localization, and size. In general, spontaneous repair of cartilage defects decreases with increasing defect size. Another factor that plays a role in the natural history of (osteo)chondral defects is an unicompartimental increase in joint contact forces, as occurring, for example, in the medial tibiofemoral compartment in patients with axial malalignment of the lower extremity, as it may accelerate degeneration.

Surgical treatment options for articular cartilage defects

The multitude of surgical interventions aiming either to replace the damaged (osteo)chondral tissue or to induce repair attest to the unsolved clinical problem of true cartilage regeneration. Marrow-stimulation techniques establish a communication between the cartilage defect and cells from the subchondral BM either by focal perforation of the subchondral bone plate with awls (microfracture), drill bits (subchondral drilling), or by a generalized abrasion of the subchondral bone plate with burrs (abrasion arthroplasty) [4]. BM from the subchondral compartment fills the defect and a three-dimensional (3D) BM clot containing mobilized pluripotent MSC forms. These stem cells are undergoing chondrogenic differentiation, resulting in the formation of a fibrocartilaginous repair tissue. For larger defect areas, clinical evidence suggests that a sufficient defect repair by this strategy is insufficient. Osteochondral autografts and allografts involve the transplantation of osteochondral tissue into areas of cartilage damage. They are chiefly indicated for small osteochondral defects such as resulting from OD. For larger defects areas, multiple osteochondral cylinders may have to be used, preferentially as osteochondral allografts because of the increasing donor-site morbidity when harvesting osteochondral autografts.

Tissue-engineered cartilage products for orthopedic reconstruction

Tissue engineering of cartilage is based on seeding chondrocytes in or onto biodegradable scaffolds for defect repair. For cartilage repair, the engineered construct is nearly exclusively used to repair focal defects of the articular cartilage. In addition to articular cartilage reconstructive applications, engineered cartilage can principally be also used in head and neck reconstruction, for example, treating defects in the auricle, nose, and trachea. For articular cartilage repair, both a sufficient horizontal and vertical integration with the underlying subchondral bone and adjacent native articular cartilage are specifically challenging. Ideally, the mechanical properties of the engineered construct should be similar to those of the adjacent cartilage in order to prevent perifocal OA. At the time of implantation, however, they are usually inferior, relying on the in situ maturation of the implant over time. Moreover, the scaffold needs to provide an environment that guarantees chondrogenic stability of the implanted chondrocytes. Although it is recommended that such an implant should also survive in an inflammatory environment, significant clinical inflammation is clinically absent as this represents a contraindication, similar to OA or arthritis such as rheumatoid arthritis (RA). Despite significant advances, however, no engineered construct is capable to completely mimic the structure and properties of the native articular cartilage. As cartilage defects occur in different sizes and are often irregular in shape, the transplanted construct needs to be either robust enough to sustain a trimming in the operation room to be implanted in a defect specific shape or to adapt itself to the defect.

Cells for tissue-engineered cartilage repair

Chondrocytes are the logical choice for tissue-engineered cartilage repair since they represent the resident cell population and are consequently capable of proliferation and cartilage-specific extracellular matrix (ECM) production and deposition. The first step in tissue-engineered cartilage repair is the biopsy of cartilaginous tissue containing chondrocytes. It is usually performed arthroscopically. Cartilage biopsies are taken from within the knee joint, even if the engineered transplant is implanted in other joints such as hip or ankle. In contrast, the use of cartilage fragments or material from debridement does not meet the requirements for a standardized collection process and is therefore discouraged. As such a biopsy represents by definition a secondary injury within the joint, other sources for chondrocytes such as rib, auricular or nasal cartilage, or allogeneic chondrocytes have been explored. Nasoseptal chondrocytes can be harvested by nasal biopsy and are currently evaluated in clinical trials for articular cartilage repair in the knee joint. Since these are heterotopic nonarticular chondrocytes, structural clinical data on the repair tissue are mandatory. Future efforts to increase their potential

include strategies reducing possible dedifferentiation and applying chondrocytes from OA cartilage.

Adult MSCs from BM, adipose tissue, adipose tissue, muscle, synovium, periosteum, perichondrium, and other sources have chondrogenic differentiation ability, thus appearing as an alternative. Nevertheless, also MSCs pose several challenges, including relatively low cell numbers within the donor tissue, the need for characterization and chondrogenic differentiation which in vitro takes several weeks, the similar instability of the differentiated chondrogenic phenotype, and unanswered question of differentiation into other lineages such as osteoblasts within a cartilage defect in vivo. Interestingly, cartilaginous constructs engineered in vitro from BM-derived MSCs have been shown to be dissimilar in their epigenetic signature to constructs from primary chondrocytes that are almost identical to autologous articular cartilage, necessitating future investigation [5].

Scaffolds for clinical tissue-engineered cartilage repair

An ideal scaffold for articular cartilage repair must provide a favorable 3D environment for the maintenance of the differentiated chondrocyte phenotype. It needs to enable attachment both of implanted chondrocytes and cells migrating into the defect. The scaffold must allow the diffusion of nutrients and chondrogenic factors as well as allow the removal of cellular waste products. It should be biodegradable, ranging from an initially biomechanically strong support which is reduced over time in a rate paralleling ECM deposition and tissue remodeling. An abundance of natural, synthetic, or hybrid scaffold materials have been developed and tested for tissue engineering of cartilage in vitro, and in animal models in vivo. As only a few have successfully found entry into clinical applications, a focus is placed on such materials (Table 80.1).

From a surgical standpoint, an application via an arthroscopic procedure would be desirable. Solid scaffolds are attractive because their initial mechanical stability immediately upon implantation makes them potentially interesting to treat large cartilage defects. Currently, the majority of scaffold materials in clinical applications are based on solid materials. However, hydrogel scaffolds are ideal candidates for implantation by minimally invasive arthroscopic surgery. They can be tailored to exhibit similar mechanical, but also swelling, and lubricating behavior as articular cartilage. Also, hydrogels allow the seeded chondrocytes to retain their differentiated chondrogenic phenotype characterized by a spherical morphology. Remarkably, also decellularized cartilage has been studied as a suitable basis for tissue-engineered repair because of the correct assembly of the ECM macromolecules and its good mechanical properties, although the process of cellular population is not as efficient as for preseeded or porous scaffolds.

Collagen scaffolds

Collagen scaffolds are of either natural or synthetic origin, or hybrids. Natural scaffolds such as type-I collagen are currently the most widely used to repair cartilage in clinical applications. Although type-I collagen is associated with the fibrocartilaginous phenotype of the cartilaginous repair tissue, it often shows a mixed morphology containing both types-I and -II collagens. Type-III collagen functions in the healing response of cartilage damage as a covalent modifier to add cohesion to a weakened type-II collagen network. Most of the commonly used type-I/III collagen membranes are derived from animals. The collagen molecules in these scaffolds are cross-linked to strengthen the fibrils, stabilize their structure, extend resorption time, and enhance biocompatibility. Commercially available type-I/III collagen membranes for applications in articular cartilage repair usually comprise a bilayer structure. The layer facing the synovia (and the opposing cartilage) is compact and has a smooth surface with a low porosity. The other (inner) layer is more porous and has a spongy 3D appearance. While

TABLE 80.1 Overview of scaffolds for clinical tissue-engineered cartilage repair.

Material	Structure of the three-dimensional network	Example of commercial products
Type-I/III collagen	Solid scaffold	MACI, Novocart 3D
Type-I collagen (atelocollagen)	Hydrogel	Koken Atelocollagen Implant
Hyaluronic acid	Solid scaffold	Hyalograft C
Hyaluronic acid	Hydrogel	CARTISTEM
Albumin and hyaluronic acid	Hydrogel	Novocart Inject
PGA, PLA, and polydioxanone	Solid scaffold	BioSeed C
Fibrin	Hydrogel	Chondron
Agarose and alginate	Hydrogel	Cartipatch

Note that not all commercial products (e.g., Hyalograft C) are currently available. Only scaffolds that have been used in conjunction with cells are shown.
PGA, polyglycolic acid; *PLA*, polylactic acid.

most type-I/III collagen products are solid scaffold membranes, type-I collagen (atelocollagen) is also used as hydrogel scaffold for ACI. Type-II collagen is the principal molecular component of cartilage and plays a key role in maintaining chondrocyte function. Not as easy available as type-I collagen, recombinant human type-II collagen has been experimentally used in vitro and in vivo for tissue engineering. Although comparative studies revealed the superiority of type-II collagen for chondrocyte phenotypic stability versus type-I collagen, hydrogels based on type-I collagen (as also alginate and agarose) also allow to maintain the chondrocytic phenotype. As these collagens are rapidly remodeled in vivo, their main task is to provide a temporary matrix within the cartilage defect to support the transplanted cells, rather than acting as a structural matrix that is present for a long time. Other cartilage-specific collagens such as types-VI, IX, X, XI, XII, and XIV found little use in tissue engineering approaches so far.

Hyaluronan

Hyaluronan (or hyaluronic acid) is a naturally occurring glycosaminoglycan (GAG). Hyaluronan is an important component of the articular cartilage ECM. It also acts to lubricate the articular cartilage surfaces in synovial joints. In addition, hyaluronan binds to the aggrecan molecules in the ECM, forming larger aggregate structures. It has been used for cell delivery both as a solid scaffold or hydrogel.

Synthetic polymers

Synthetic polyesters such as poly(lactide acid) (PLA), poly(glycolid acid) (PGA), and their poly(lactide-*co*-glycolide) (PLGA) copolymers are the most often used biodegradable polymers. Devices made of PLGA combine good mechanical properties and defined degradation according to the specific tissue requirements. They can be easily extruded into fibrous or open lattice structures, ideally supporting cell attachment, and have been extensively used to engineer cartilage in vitro and in vivo. Self-assembling peptides are another versatile class of materials capable of providing a microenvironment to enhance chondrogenesis. These peptides are able to self-assemble into stable hydrogels at low peptide concentrations but have not been tested in clinical settings.

Agarose and alginate

Both agarose and alginate are natural carbohydrate polymers that are manufactured from marine algae. Agarose is a linear polymer based on a disaccharide consisting of made up of D-galactose and 3,6-anhydro-L-galactopyranose. Alginate is a linear copolymer with homopolymeric blocks of β-D-mannuronate and α-L-guluronate at different concentrations. Both polymers provide a 3D environment for chondrocytes, supporting their differentiated phenotype and have been applied for tissue engineering purposes. They may be combined with other materials, such as chitosan. Although translational studies attest to the potential value of agarose- or alginate-based hydrogels for chondrocyte delivery into cartilage defects, clinical applications have been rare [6].

Scaffold-free three-dimensional systems

Scaffold-free 3D systems have also found entry into clinical applications, consisting of chondrocytes as spheroids. The chondrocytes are cultivated in culture plates where they cannot adhere and consequently start to aggregate into a round high-density structure, mimicking the cell condensation observed during the embryonic development of cartilage. Inside the spheroids, the chondrocytes display a round cell shape. As the synthesis and deposition of ECM by the chondrocytes continues within the spheroids, the chondrocytes progressively encapsulate themselves within a self-synthesized ECM. The ECM is composed of types-I and -II collagens, GAGs, and aggrecan, comparable to an early chondrogenic phenotype. Interestingly, more type-I collagens is deposited near the surface of the spheroids, where the cells have an elongated shape. The chondrocytes in the spheroids express chondrogenic growth factors, which are insulin-like growth factor I, transforming growth factor beta, bone morphogenetic proteins-2 and -4, and platelet-derived growth factor. Such growth factor expression is important for the success of all ACI technologies as this suggests a continuing paracrine effect of the transplanted cells within the cartilaginous repair tissue in vivo.

Bioreactors for tissue-engineered cartilage repair

Considering the fact that the phenotypic stability of articular chondrocytes can be supported by using specific environmental factors such as 3D culture, low oxygen tension, and mechanical stimuli, bioreactors represent a logical choice for their cultivation for clinical protocols. Culture of chondrocytes in 3D bioreactors has long been a powerful tool to enhance the structural and functional properties of tissue-engineered cartilage. Such constructs, cultivated in rotating bioreactor systems, are characterized by a more uniform cell seeding, increased cell numbers, and enhanced ECM deposition compared to static cultivation systems. Such increased structure properties directly translate to increased load-bearing capacities and stiffness by the tissue-engineered cartilage. Yet no current clinically available protocol does apply these advances. Nevertheless, it

may be speculated that in the future such bioreactor-based strategies will find also applications in commercial manufacturing strategies for chondrocyte implantation.

Clinical nomenclature of scaffold-based techniques

Clinical generations of autologous chondrocyte implantation

The clinical nomenclature of ACI generations reflects the evolution of the technique. It spans from the use of a periosteal flap to biomaterial scaffolds that are preseeded with cells. The initial first-generation ACI procedure as proposed by Grande, Peterson, and Brittberg is based on the inoculation of a cell suspension containing expanded autologous chondrocytes into the hollow space that is created by covering the previously prepared cartilage defect with a biological membrane of autologous periosteum removed from the ipsilateral proximal tibia. The first generation of ACI focused on safe and effective cell application. Harvesting of the flap was challenging and time-consuming, but most importantly the spatial distribution of the implanted cells was irregular. As undesired effects such as hypertrophy of the periosteal graft and failures were noted [7], biomaterial scaffolds were introduced as substitutes for the periosteal flap, leading to "second-generation" and "third-generation" ACI. Second-generation ACI uses a biomaterial scaffold (mainly a type-I/III collagen membrane) as a cover of the defect where the cell suspension is injected in vivo in the operation room in a similar fashion than when using the periosteal cover. Third-generation ACI further applies the principles of tissue engineering as the expanded chondrocytes are already seeded into the 3D biomaterial scaffold before implantation, allowing the cells to settle down and attach on the scaffold fibers ex vivo, thereby avoiding the asymmetric cell distribution following injection of the cell suspension into a possible oblique defect base in vivo. Cell quality was optimized by characterization, minimally invasive application, focusing on a purely autologous setting without foreign material, and modifying the environment and biomaterials. Thus the nature of the structural template for chondrocyte implantation evolved from a purely biological membrane to a preseeded resorbable biomaterial that, in most cases, is a solid membrane but may also constitute a hydrogel scaffold.

Acellular, scaffold-based products

The importance of this scaffold evolution is also reflected in the clinical advent of the concepts of acellular, scaffold-based products, which chiefly aim to combine biomaterial scaffolds with marrow stimulation. Of note, these so-called matrix-augmented BM stimulation techniques do not use ex vivo cultivated chondrocytes as in the ACI procedure, and the abbreviation "AMIC" of the (trademark-protected) term "autologous matrix-induced chondrogenesis" may not be confused with MACI, another (trademark-protected) term referring to an ACI product based on a porcine type-I/III collagen membrane. Of surgical technical importance, ACI never includes the penetration of the subchondral bone plate (i.e., microfracture) which is mandatory for matrix-augmented BM stimulation techniques. A large variety of such products is on the market, including BST Cargel (chitosan), CaReS-1S (rat type-I/III collagen), Cartimaix (porcine type-I/III collagen), Chondrofiller (rat type-I/III collagen), Chondro-Gide (porcine type-I/III collagen), Chondrotissue (synthetic PGA and hyaluronic acid), Hyalofast (hyaluronic acid), and Novocart Basic (bovine type-I/III collagen) [8]. Although such matrix-augmented BM stimulation techniques appear to be clinically safe, there is a paucity of high-quality, randomized long-term controlled studies testing them versus established procedures such as microfracture or ACI especially for the knee, making it difficult to recommend joint-specific indications for this, from a biomaterial standpoint, interesting technique [9]. However, such a sole biomaterial implantation appears especially attractive as an off-the-shelf therapeutic option, serving the demand of more economical solutions for cartilage repair although caution has to be applied based on their lack of long-term clinical data. In any case, such acellular membranes may be used in salvage procedures after failed previous attempts at cartilage repair to cover large defects when ACI is contraindicated or not available.

Particulated autologous or allogenic articular cartilage

Other commercially available products involve the implantation of the natural cartilage ECM with chondrocytes in the form of particulated articular cartilage from either autograft or juvenile allograft donors, among which Cartilage Autograft Implantation System (CAIS), DeNovo Natural Tissue (NT) Graft (DeNovo NT), and Particulated Juvenile Allograft Cartilage (PJAC) neither use isolated and expanded chondrocytes nor a 3D biomaterial scaffold, with the exception of the fibrin glue that may be used to attach these cartilage fragments to the defect [10,11].

Commercial autologous chondrocyte implantation products

MACI (Vericel, Cambridge, MA, United States)

The MACI product involves the established method in which autologous chondrocytes are expanded in monolayer culture after initial enzymatic digestion. Several

days before implantation they are seeded onto on a resorbable type-I/III collagen membrane (Chondro-Gide, Geistlich, Wolhusen, Switzerland). The MACI implant represents a cellular sheet, consisting of autologous cultured chondrocytes at a density of about 500,000 cells per cm^2.

ChondroCelect (TiGenix, Leuven, Belgium)

The product ChondroCelect (TiGenix, Leuven, Belgium) was the first cartilage cell product approved as advanced therapy medicinal product (ATMP). The initial idea was, at the molecular level, to assess efficacy through the characterization of marker genes associated with chondrogenic performance which led to the term of "characterized chondrocyte implantation" of the product. Nevertheless, the technique and the surgical application are not different from the first and second-generation ACI products. Even though the initial approval was submitted and granted for the combination with an autologous periosteal flap, a porcine type-I/III collagen membrane was recommended. The innovation of the product has to be seen in the field of cell biology: The chondrocytes cartilage cells are characterized in vitro in terms of chondrogenic potency. Yet, it should be noted that other manufacturers are also obliged to perform an analysis of chondrogenic markers as a prerequisite for the detection of the quality of the product, and the characterization of chondrocyte marker gene expression did not have a clinical consequence. From a clinical point of view, a structural superiority after 1 year in direct comparison with microfracture, a functionally better treatment result after 2 years, and in the subgroup of patients with a short symptom duration, a superiority after 5 years all have been demonstrated.

Spherox (Co.don, Berlin, Germany)

Spherox represents the concept of a scaffold-free 3D system, including spheroids of autologous chondrocytes within their ECM. The chondrocyte cultivation takes place in two steps: after isolation of the chondrocytes, their propagation is performed first in monolayer culture using autologous patient serum and growth factor-free medium. Subsequently, the cells are transferred into a 3D culture as spheroids. Already in this phase of cultivation, the chondrocytes produce and deposit an ECM. A spheroid contains approximately 200,000 cells and measures about 300−600 μm in diameter at the time of implantation. They are directly applied either arthroscopically or via arthrotomy to the subchondral bone plate (about 10−70 spheroids/cm^2 defect) in a dry environment where they adhere to the host within about 20 minutes and obtain a flattened shape. The adhesion and subsequent in situ remodeling are considered crucial steps for its integration into the defect. It consists of an initial fixation phase by cell mediated adhesion to the host subchondral bone, continuous integration via migration of the transplanted chondrocytes along the irregular surface of the subchondral bone plate and spheroid remodeling. A biomaterial coverage or fixation is not required, supporting the minimally invasive application.

Novocart 3D (Tetec, Reutlingen, Germany)

The product Novocart 3D represents a classic representative of matrix-associated ACI using a biphasic biomaterial. Novocart 3D is distributed since the end of 2003. Here, the articular chondrocytes are already preoperatively seeded in a type-I/III collagen-based biphasic matrix in a density of 0.75 to 4.0×10^6 cells/cm^2 matrix. The bovine biphasic matrix consists of a covering membrane and an underlying collagen sponge, whose interconnecting pores allow a homogeneous 3D cell distribution. Implantation of Novocart 3D typically requires a (mini) arthrotomy to allow secure fixation of the support membrane with sutures to the surrounding cartilage. For defects where stable edge walls cannot be created, resorbable pins are an alternative.

BioSeed C (Biotissue, Geneva, Switzerland)

The product BioSeed C represents one of the first representatives of the so-called third generation of ACI, which combines in vitro the expanded chondrocytes with a synthetic polymer-based biomaterial as a carrier for before transplantation. It is distributed since 2002. For cell seeding, a porous fleece based on PGA, PLA, and polydioxanone, on which chondrocytes are suspended in fibrin glue, is used. The material can be tailored according to the defect size. The fleece is fixated with sutures to the surrounding cartilage, or with transosseous sutures or arthroscopic darts. It should be noted that this product has not yet initiated a central approval by the European Admission Office (EMA) in the sense of initiating a registration study.

Novocart Inject (Tetec, Reutlingen, Germany)

The product Novocart Inject pursues a technically different approach as cells are applied in a hydrogel in contract to nearly all other approaches which are based on solid scaffolds. Novocart Inject is an injectable, chondrocyte-containing hydrogel on the basis of albumin and hyaluronic acid that polymerizes in the defect after being combined with a biocompatible cross-linker similar to fibrin glue. Novocart Inject also reaches defect locations that are difficult to address. It can be applied using arthroscopic techniques in a dry environment, or by mini-arthrotomy. Registration trials are already initiated for Novocart Inject and the first patients were enrolled in 2018.

Chondron (Sewon Cellontech, Seoul, Korea)

Chondron is a technique based on the transplantation of in vitro cultured autologous chondrocytes which are embedded in fibrin glue as hydrogel scaffold and then implanted into the cartilage defect. Case series without control groups have been reported [12].

Cartipatch (Tissue Bank of France, Génie Tissulaire, Lyon, France)

Cartipatch is a solid agarose/alginate hydrogel scaffold containing autologous chondrocytes. Following monolayer culture for 3 weeks, cells are placed in the hydrogel matrix to produce cylindrical hydrogel grafts of 10, 14, and 18 mm in diameter. A multicenter randomized controlled trial reported significantly inferior functional outcomes compared to mosaicplasty for isolated focal osteochondral defects of the femur at 2 years postoperatively [6].

CARTISTEM (Medipost, Seongnam, Korea)

CARTISTEM is the first allogeneic stem cell product for the treatment of knee cartilage defects in patients including ICRS grade IV OA lesions approved in South Korea. It is based on allogeneic human umbilical cord blood (hUCB)−derived MSCs (hUCB-MSCs) given at a dosage of 2.5×10^6 cells/500 μL/cm^2 cartilage defect area. The stem cell−based medicinal product is a composite of culture-expanded allogeneic hUCB-MSCs and a hyaluronic acid hydrogel. hUCB is collected from umbilical veins at the time of neonatal delivery, stored in a cord blood bank, and isolated and characterized hUCB-MSCs are cultivated in medium supplemented with 10% fetal bovine serum, maintained in monolayer culture for approximately 2 weeks and resuspended in a hyaluronic acid hydrogel when reaching 80% confluence. Using a standard arthroscopic procedure followed by a mini-arthrotomy, the cartilage defect is first treated with prograde subchondral multiple drill holes (5 mm in diameter, 5 mm deep, approximately 2−3 mm apart). Next, the allogeneic hUCB-MSCs/hydrogel composite is implanted in each of the drill holes [13].

JACC stands for a product based on isolated autologous chondrocytes that are cultured in a 3D atelocollagen gel environment [Japan Tissue Engineering Co, Ltd. (J-TEC) autologous cultured cartilage (JACC) system]. Following cell isolation, the cells are mixed with an atelocollagen solution containing 3% type-I collagen. The final cell density per the hydrogel depends on the size of the cartilage defect. The cultured hydrogel is applied to the defect and covered with a flap of periosteum harvested from the femoral condyle.

Clinical application of autologous chondrocyte implantation in reconstructive articular cartilage surgery

Indications for autologous chondrocyte implantation

The value of ACI lies primarily in the treatment of large, symptomatic, full thickness focal non-OA chondral or osteochondral defects in the knee joint in patients ≤ 50 years. For cartilage defects in children and adolescents, ACI is not primarily recommended due to their good intrinsic repair capacity. There is a high degree of agreement in international guidelines and recommendations, reflecting the limited success of BM stimulating techniques for large-area cartilage damage. A structural superiority of the repair tissue following ACI has been shown in prospective randomized studies. Through sequential international recommendations, the indications for ACI have been refined, especially the lower limit of defect size, for which an ACI is recommended, especially in young and physically active patients. ACI is currently indicated for primary defects with areas above 2.5 cm^2. For other patients, there is a consensus of treating defects with areas above 3.0 cm^2 with ACI. Another indication is defects of all sizes in the same age group, in which previous cartilage-repairing measures failed. The cartilage of the corresponding articular surface may be damaged up to a maximum of II in the International Cartilage Regeneration & Joint Preservation Society (ICRS) classification (excluding ICRS grades III or IV; ICRS Cartilage Injury Evaluation Package 2000). A reduction of the total ipsilateral meniscus volume of a maximum of one third is permitted. It is important to recognize that a physiological leg axis must be present or possibly created, since analyses of failed cases revealed local overload based on untreated axial malalignment as the most commonly recognized reason (in 56% cases) for failed surgical index procedures such as microfractures or osteochondral transplantations. For example, in the cases of patients with focal cartilage lesions that are located in the medial femoral condyle with accompanying varus deformities of >5 degrees, there is general consensus to realign such deformities together when performing an ACI either in a one- or two-staged scenario. The evidence for the upper ankle or hip joint is much lower, and for other joints (glenohumeral) only a few case reports exist.

Contraindications

ACI is not indicated for the treatment of advanced OA [Kellgren−Lawrence (K−L) grade 3 or 4]. Other specific contraindications include conditions such as hypersensitivity

to products of porcine or bovine origin and inflammatory arthritis including RA.

Surgical steps

The first step of the surgical procedure is the strict examination of the knee joint, which needs to be performed arthroscopically in analogy to the current international recommendations. If the indication for ACI is confirmed intraoperatively, the arthroscopic procedure for the biopsy of cartilaginous tissue can directly follow. The most common method is the removal of three defined narrow osteochondral cylinders using small hollow punches from regions of normal cartilage. The superolateral or superomedial trochlea and the superior border of the intercondylar fossa are suitable locations. By definition, this removal process represents the first step in the manufacture of a drug within the context of "ATMP" and is thus subject to regulation. Such regulation includes a standardized collection process for the removal of cartilage, the examination of the donor suitability, careful documentation of the biopsy process, regular instructions of the responsible persons, and fulfillment of structural and hygienic conditions, together with documentation and filing of all documents for 30 years after collection.

After submitting the osteochondral cylinders in cell culture medium to an approved laboratory by express delivery, the enzymatic digestion of the cartilage which has been separated from the subchondral bone and the primary monolayer culture of the isolated articular chondrocytes in a clean room laboratory are carried out. Subsequently, the monolayer expansion of the chondrocytes takes place in the context of a standardized proprietary expansion protocol of the cooperating company and the production of the final product. This period varies depending on the manufacturer between about 3 and 8 weeks. It is not influenced by the surgical demands, solely depending on the manufacturing process. The possibility of an interim cryopreservation of the chondrocytes makes it possible to adapt the time of transplantation to the needs of the patient. This option is offered by most commercial ACI suppliers.

Following cell expansion, chondrocytes are seeded into the biomaterial scaffold, which will be implanted in most cases. This is performed either in the laboratory some days before implantation or a cell suspension is being delivered directly to the operation theater where the suspended chondrocytes are seeded into the biomaterial immediately before implantation. As an alternative, 3D spherical aggregates of chondrocytes obtained in a similar fashion may be implanted without the use of a scaffold into defects.

Implantation of the ACI product may be performed as open surgery (arthrotomy) or arthroscopically, chiefly depending on the type of product (Fig. 80.1). In most cases, an arthrotomy is needed. A meticulous surgical technique has to be applied. After defect identification, the careful debridement of the diseased cartilage tissue represents the first surgical step. In contrast to marrow-stimulation techniques, the integrity of the subchondral bone plate is preserved. During debridement, all diseased cartilage tissue ultimately has to be removed, resulting in stable and vertical defect walls surrounded by healthy and vital cartilage and a subchondral bone plate lamella free from residual calcified cartilage tissue, avoiding bleeding from the subchondral bone, as in vitro studies show a negative influence of blood on the regeneration capacity. The technique of implantation and fixation is product-dependent. In general, the supporting membrane is accurately adapted to the geometry of the defect, implanted and firmly fixed (Fig. 80.2). For fixation, it may be anchored to the adjacent cartilage using single interrupted sutures (e.g., USP 6-0) or with resorbable pins or fibrin glue (Fig. 80.3). Erosion of the subchondral bone plate is tolerable up to a depth of the bony lesion of about 5 mm. Deeper osteochondral defects should be recontoured by filling with autologous cancellous bone. This "sandwich technique" is a useful option for large osteochondral defects based on osteochondritis dissecans (OD), where there is no possibility of fragment replication.

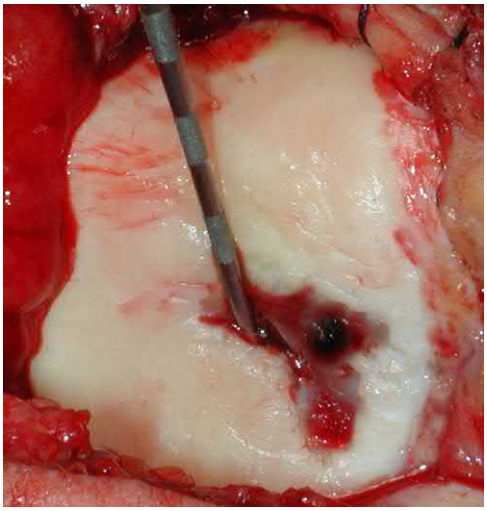

FIGURE 80.1 Cartilage defect of the patella in a 28-year-old man as a result of a direct trauma in the course of a traumatic patellar luxation. Note the partial subchondral involvement indicated by the incomplete blood clot at the base of the defect.

Clinical results of autologous chondrocyte implantation

Overview

A large clinical body of therapeutic evidence already exists for ACI (Fig. 80.4). Long-term studies with a

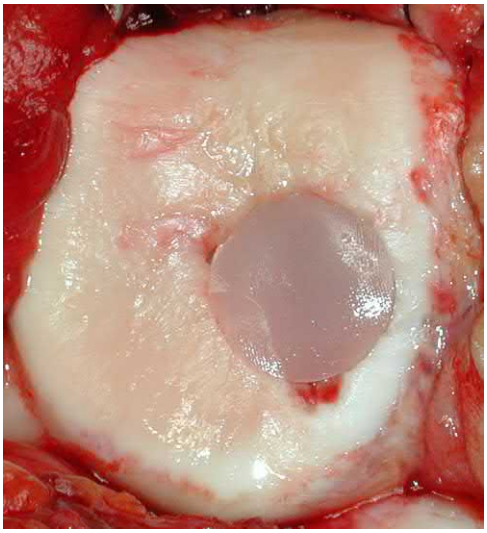

FIGURE 80.2 After meticulous defect preparation, the membrane supporting the chondrocytes in this third-generation ACI product is accurately adapted to the geometry of the defect and implanted. *ACI*, Autologous chondrocyte implantation.

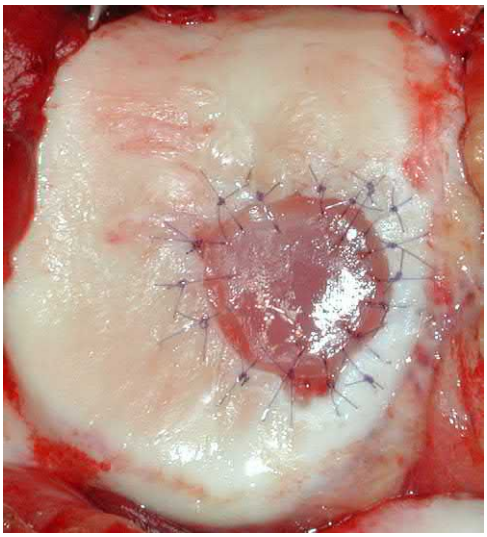

FIGURE 80.3 Fixation of the ACI membrane to the adjacent cartilage with single interrupted sutures (USP 6-0) has been performed. *ACI*, Autologous chondrocyte implantation.

follow-up of up to 20 years, specific information on typical complications, together with meta-analyses of several thousand patients on its clinical results and the important possibility of returning to sports for the younger patient population that mainly benefits from the intervention, all showing relatively good outcome parameters. Most importantly, a significant number of data from prospective randomized clinical trials (RCTs) have refined the indications for ACI, especially within the context of the regulatory steps as required for approval of ACI as a drug. Most of these very valuable studies have been designed for ethical reasons as "noninferiority studies,"

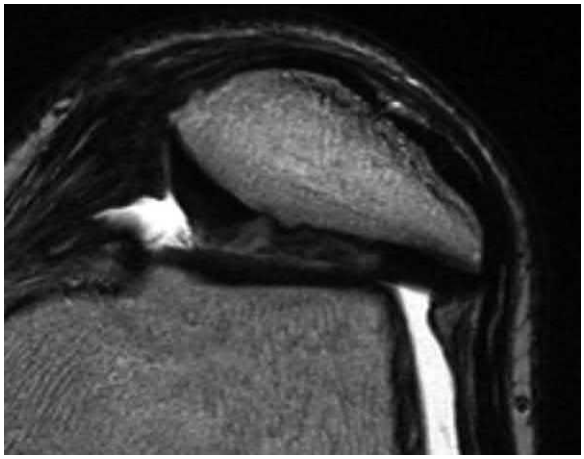

FIGURE 80.4 MRI of the femoropatellar joint of the case after 1 year. Note the complete filling of the defect with a repair tissue, its relatively good integration to the adjacent articular cartilage and the subchondral bone, the irregular subchondral bone plate and the structural differences of the repair tissue compared to the adjacent cartilage.

thus the control group represents another form of surgical therapy, which is in the most of the cases an arthroscopic microfracture. However, in the context of these studies, it has to be kept in mind that most of these studies comparing arthroscopic microfracture with ACI are studying patients with defect sizes that are rather small, thus often not meeting the indications for ACI, which are chondral defects larger than $2.5-3.0\,cm^2$ (marrow stimulation is indicated for defects smaller than $2.5-3.0\,cm^2$). As the defect size is an essential criterion, a direct comparability with microfracturing for larger defects has not been performed to date. The fact that most long-term RCTs are based on defects sizes not higher than $4.0\,cm^2$ and involve first-generation ACI suggests that more high-quality clinical evidence is needed for a satisfactory answer as to whether recent techniques of ACI show superior clinical outcomes in long-term follow-up compared with microfracture. Nevertheless, meta-analyses of the second and third generations of ACI (i.e., the currently marketed products) show the evidence of superiority of ACI in individual studies at the structural (histology) and clinical levels.

Data from prospective randomized clinical trials

Knutsen et al. reported a long-term follow-up at 15 years of a randomized multicenter trial (level I) comparing first-generation ACI with microfracture. The 80 patients had cartilage defects mainly in the femoral condyles, and the defect sizes ranged from 1.4 to $11.2\,cm^2$. At 15 years, the clinical data from this important trial showed significant clinical improvements compared with baseline (clinical scores and pain) and no significant differences between both treatment groups. Failures were noted in

both groups (ACI; $n = 17$, microfracture; $n = 13$), and sometimes total knee replacements were needed (ACI; $n = 6$, microfracture; $n = 3$), without significant differences between them. Of note, about 50% of the surviving patients in both groups had radiographic evidence of early OA (e.g., a K−L grade of ≥ 2) without significant differences between the groups, showing that the problem of delaying OA has not been solved [14]. Although this clinical trial applying a first-generation technique did not show superiority of ACI versus marrow stimulation, there is a strong consensus in the international clinical cartilage repair community that ACI is beneficial to treat either large lesions or as a rescue technique following failed other previous repair strategies. A recent systematic review of 5-year outcomes comparing microfracture versus ACI for articular cartilage lesions in the knee showed that patients undergoing microfracture or first/third-generation ACI can be expected to experience improvements in clinical outcomes at midterm to long-term follow-up without any significant difference between the groups [15].

Long-term results of autologous chondrocyte implantation

In addition to evidence-based prospective studies, long-term results for the treatment of cartilage defects in the knee joint with ACI are now available. Although these represent uncontrolled studies and thus cannot provide any statements about a direct superiority of the method over other treatment options, they may serve as a general proof of the long-term efficacy of the surgical method. At a mean follow-up of 10.9 ± 1.1 years, first-generation ACI led to satisfying clinical results (patient satisfaction, reduction of pain, and improvement in knee function). Nevertheless, full restoration of knee function cannot be achieved. Interestingly, the level of structural cartilage repair as assessed using MRI did not correlate with the long-term clinical outcomes [16]. A 20-year follow-up of first-generation ACI showed satisfactory survival rates and significant clinical improvements [17].

Clinical factors affecting the clinical outcomes of autologous chondrocyte implantation

Several factors affect the clinical success of ACI besides defect size, among which depth of lesion, anatomic defect location, and patient age and occupation. Deep osteochondral defects are challenging, such as occurring after OCD or avascular necrosis, because patients suffering from these lesions have a high risk of OA if the articular congruence is not restored. Here, ACI is combined with autologous bone grafting, termed "sandwich" technique. Treating symptomatic deep osteochondral lesions in the knee was significantly better in the ACI sandwich group than in comparison with autologous bone grafting alone in terms of survival rate at a mean follow-up of 16 years. Especially no significant OA progression from preoperatively to a mean of 5 years postoperatively was seen in the ACI sandwich group based on K−L grading [18]. Since patients with cartilage damage are typically young with high levels of activity, the rate of return to exercise and the intensity with which sport can be performed again after surgery is a relevant parameter for evaluating different therapies. Return to low- and moderate-intensity levels of sport appears realistic in the majority of cases, whereas the likelihood of returning to activities with high stress applied on the knee joint is low. Neither defect location nor size appears to significantly influence postoperative sports activity or return-to-work rates [19]. Meta-analyzes based on a significant number of included cases reveal the highest rates of return to sport postoperatively if defects were treated with autologous osteochondral transplantation or ACI, while the best rate was observed in terms of the intensity of postoperative exercise capability in ACI. Yet, autologous osteochondral transplantation and microfracture (usually indicated for smaller defects) allow for a faster return to sports activity in direct comparison to ACI, although clinical performance of microfracture appears to be inferior. However, it has to be kept in mind that that postoperative sport ability is a very heterogeneous parameter, as recent data shows differences in terms of sports ability depending on the desired intensity and scope. Most importantly, the ability to return to sports and the intensity of sports do not correlate with the structural quality of the repair tissue in the postoperative course. The premorbid level of sporting and recreational activities in general cannot be achieved as shown at 11 years after first-generation ACI. Also, MRI data do not correlate with long-term sporting activity [20]. Data on anatomic defect location usually indicate that defects in the femoral condyles show superior outcomes than patellofemoral defects. Recent information from a prospective cohort of patients indicates that second-generation ACI for patellofemoral lesions successfully allowed returning to work in the majority of patients of moderate to very heavy occupational demand with significantly decreased knee pain at 2- [7] and 4-year follow-ups, adding to the evidence that ACI is a valuable cartilage repair technique for patellofemoral lesions [21]. In contrast to other cartilage repair techniques, patients 40 years and older do not have an inferior outcome up to 2 years after ACI for isolated cartilage defects when compared with younger patients [22].

From a clinical and scientific point of view, ACI has established itself in the treatment of large cartilage damage to the knee joint. The data on ACI in other joints are much more reduced in scope and value compared to the knee joint. For example, ACI is more commonly performed in the hip joint and the upper ankle joint, but the proof of its efficacy in other joint-based controlled randomized studies is still pending. Open questions are the

optimal cell dose needed for optimal morphological repair after ACI [23], and the lack of correlation between cartilage repair tissue ultrastructure and clinical or qualitative MRI outcomes [24]. Finally, it remains to be seen whether other cell sources such as MSCs are capable of achieving similarly effective MRI scores and qualitative histological and clinical outcomes [25].

Conflict of interest

The author declares no conflicts of interest in this contribution.

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Chapter 81

Bone tissue engineering

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Introduction

Bone is both a tissue and an organ. Bone defects can result from congenital anomalies, trauma, metabolic diseases, infections, and tumor removal [1,2]. Bone deficiencies represent formidable clinical challenges and have inspired creative scientific approaches for repair and regeneration. Bone tissue engineering remains one of the most robustly pursued fields in tissue engineering.

Contrasting to several other skeletal tissues, bone has certain intrinsic regenerative capabilities for self-repair. However, numerous bone defects do not heal and have been termed "critical size bone defects" and require surgical interventions [3,4]. For example, the clinical management of a critical diaphyseal defect, a large discontinuity in bone that cannot spontaneously regenerate, continues to be a major challenge. The two available treatment options are limb salvage or amputation, leading to disability [1,2]. These clinical scenarios can be complicated further by infections and/or nonunion fibrosis. Moreover, these problems and the high morbidity and associated costs of care are not limited to the civilian population but represent a major concern in the ever-increasing nonlethal combat casualties [5]. The treatment of diaphyseal bone defects has advanced over the years from amputation to reconstruction with bone grafts (vascularized, cancellous, autologous, allograft) to distraction osteogenesis with Ilizarov devices to the emergent translational possibilities of tissue engineering and regenerative medicine [6].

Similarly, oral cancer is a major reason for mandibulectomy and maxillectomy, and major neurosurgical procedures typically involve craniotomy. In vivo bone formation is a major challenge in various craniofacial reconstructions and dental procedures that involve jawbone [7,8]. Currently, major mandibular defects can be repaired with an autologous vascularized bone from a variety of donor sites, including fibula, scapula, iliac crest, or rib. A major disadvantage of this technique is that harvesting these grafts always creates a skeletal defect at the donor site and induces significant secondary morbidity. Other therapeutic options, including allografts, xenogenic tissue grafts, synthetic materials, or prosthesis, unfortunately do not yield consistent and efficient regenerative repair and are fraught with complications [9]. Significant progress in tissue engineering could yield more favorable outcomes than current clinical approaches used to repair craniofacial defects.

Conventional bone tissue engineering strategies: cells, scaffolds, and biofactors

Regenerative medicine approaches based on engineering cells and biomaterial scaffolds into "spare part" tissues promise to shape the future of reconstructive surgery and organ transplantation. To date, the use of growing *functional* engineered tissues in vitro for subsequent implantation into tissue defects in vivo remains experimental, despite some early clinical successes [10]. In this approach, combinations of cells and bioactive molecules are seeded onto three-dimensional (3D) biomaterial scaffolds [11–15]

Cells can be retrieved from a variety of sources, including embryonic stem cells, postnatal and adult stem/progenitor cells, or the most recently discovered induced pluripotent stem cells. The common approach in engineered tissue regeneration has been to isolate cells from tissue biopsies or aspirates, manipulate them, and reintroduce them into the host [14]. For bone regeneration,

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multiple cell sources have been investigated, including fresh bone marrow aspirates [9]; purified, culture-expanded bone marrow mesenchymal stem/progenitor cells [15], osteoblasts and cells that have been modified genetically to express osteogenic factors such as rhBMP [16], umbilical cord blood cells [17], adipose-derived stem/progenitor cells [18], or embryonic stem cells [19]. Perceived advantages and disadvantages of these cell sources in bone tissue engineering have recently been reviewed [8].

One of the pivotal challenges of cell transplantation is the cost and complexity associated with the development of experimental strategies into regulatory approved products. Intraoperative cell processing, while immune from regulatory approval, can only serve as a *point-of-care* service for one patient at a time. Once the cells are manipulated off site, regulatory approval is automatically required. Cell transplantation has encountered a number of barriers toward clinical translation, including potential immune rejection for nonautologous cells, pathogen transmission, potential tumorigenesis, costs associated with packaging, storage and shipping, shelf life and reluctance of physicians, and insurance in clinical adoption [8]. Cell survival in the host is also an unsettled issue, regardless of the cell source, and there is debate on whether the transplanted cells are regenerative per se or simply act as a pleiotropic source of factors and signals, especially in their ability to regulate inflammation [20]. These barriers will continue to be challenges for the implementation of engineered bone as a clinical treatment in foreseeable future. An alternative paradigm is to activate endogenous stem cells to participate in bone regeneration. A case in point is periosteal progenitor cells that are activated by injury and play an indispensable role in fracture repair [21,22]. Whether simple mobilization and homing of endogenous stem cells to the defect site will suffice for regeneration and have advantages over exogenous cell transplantation remains to be proven.

The assembly of the cells into the required 3D form of the bone defect requires a scaffolding biomaterial that delivers and retains the cells, and potentially stimulates and guides their induction of tissue regeneration. The minimum requirements of biomaterial scaffolds in addition to sustenance of *form* (3D shape and size) include *fixation* (securing the attachment to the host bone and minimization micro-motion), *function* (establishment of temporary or permanent mechanical load bearing), and *formation* (provision of appropriate porosity for mass transport, revascularization, osteoinduction, and osteoconduction) [23]. Additional biocompatibility characteristics must also be met in biomaterial scaffolds, including the lack of immunogenicity and toxicity. Furthermore, scaffolds can be enhanced by surface functionalizing to elicit affinity to cell binding and interactive modulation of the cells' response and can be designed for localized, controlled delivery of various bioactive molecules.

Scaffolds can derive from native tissues and biological polymers and/or synthetic polymers and can be fabricated using a variety of conventional techniques (reviewed in [24]). Among these techniques, solid freeform fabrication offers distinct advantages in enabling exquisite control of the scaffold form and internal architecture based on medical image-guided 3D modeling of the bone defect [25]. Recently, 3D bioprinting has been enabled by the commercial availability of low temperature, high resolution, multiinjector 3D-printing systems, which were originally developed for rapid prototyping applications. This technology has been successfully adapted for bone tissue engineering with biocompatible and osteoinductive calcium phosphate powder and biocompatible binder system for computed tomography (CT)-guided 3D printing of patient-specific scaffolds [26]. The multiinjector capabilities of a *colored* 3D printer potentially allow the embedding of combinations of biofactors and molecules within the scaffold with spatial control, which can be attractive in scenarios that might require spatiotemporal control over release kinetics. However, a recent review of scaffolds for bone tissue engineering has painted a bleak picture for translational progress of the field [23], which remains riddled with technical challenges of designing, manufacturing, and functionalizing scaffolds, regulatory approval barriers, business challenges related to meeting identifying niche markets and generating large initial investments necessary to sustain the business through the long-drawn-out regulatory process, and intellectual property life cycle issues that must protect the product long enough beyond the regulatory process to recoup the investment and make these products commercially viable.

Delivery of molecules and/or scaffolds to augment endogenous bone regeneration

Delivery of biofactors and molecules can alter cell signaling in the defect milieu and has been shown to influence the outcome of regeneration. A popular paradigm in tissue engineering suggests that reactivating developmental factors and signaling might be necessary for true regeneration of the lost adult tissue [27]. However, it is unclear whether the complex developmental signaling gradients and cascades need to or can be replicated faithfully in postnatal tissue repair. Regardless, our understanding of the developmental biology of the musculoskeletal system, and more specifically endochondral and intramembranous bone formation in the embryo, provides us with a plethora of information about factors, which when applied individually can enhance bone regeneration. This latter, a simpler approach is preferable for therapeutic translation.

A case in point has been the discovery of bone morphogenetic proteins (BMP) that were discovered by Marshall Urist and touted for their osteoinductive properties [28–30].

Basic science studies using transgenic mice in which individual BMPs have been selectively knocked out from the limb skeleton have identified BMP-2 as a critical factor in the innate regenerative capacity of bone [31]. The combination of recombinant human BMP-2 on an absorbable collagen sponge (ACS) carrier has been one of the most studied systems in preclinical and clinical investigations and represents one of the most significant therapeutic orthopedic discoveries [32]. With supporting level 1 clinical trial data, rhBMP-2/ACS (INFUSE Bone Graft) is commercially available, at the time of this writing, for three Food and Drug Administration (FDA)-approved clinical indications, including spinal fusion, open tibial fractures with an intermedullary (IM) nail fixation, and oral and maxillofacial augmentation (sinus augmentations and alveolar ridge augmentations for defects associated with extraction sockets) [32]. However, the efficacy of INFUSE Bone Grafts requires supraphysiological concentrations of BMP-2, and numerous adverse events have been filed at the FDA and reported in the literature in approved indications and off-label uses [33,34]. Therefore the identification of effective doses of BMP-2 (and perhaps other osteogenic and vasculogenic factors) for the regeneration of critical bone defects, preferably with tolerable and subclinical side effects, remains a common challenge for tissue regeneration community.

An exciting prospect has been the discovery of the therapeutic value of systemic hormones such as parathyroid hormone in fracture repair [35–38] and tissue engineering of critical diaphyseal defects in preclinical models [39–41]. This systemic delivery approach might overcome the challenges associated with local delivery but remains to be clinically validated.

To sum, each of the individual components of the conventional tissue-engineering triad (cells, scaffolds, and biofactors) brings unique sets of challenges. Optimization of these *composite* constructs into *functional* tissue substitutes is typically performed empirically in the laboratory ex vivo using cell and tissue culture models and in vivo using preclinical animal models. However, this approach has faced difficult barriers to translation from the bench to the bedside. A three-component medical product would have at least 3^n_i possible combinations of independent variables (where n_i is the number of possible variables associated with the ith component of the three-component product), which makes the feasibility of testing the experimental matrix in a comprehensive investigation impossible. This has limited advances in the field to only incremental discoveries, despite exciting developments and breakthrough technologies that have been reported in small animal and preclinical models. The regulatory requirements of the multicomponent bone regeneration products have hindered and continue to slow down clinical translation. Nevertheless, innovative *point-of-care* regenerative approaches guided by the tissue engineering paradigm have been reported in the clinical literature with remarkable early successes.

Biomaterials development and three-dimensional printing

Clinical successes and opportunities in regenerative repair of diaphyseal defects

Segmental diaphyseal defects are common in long bone in scenarios involving tumor resection, trauma, and

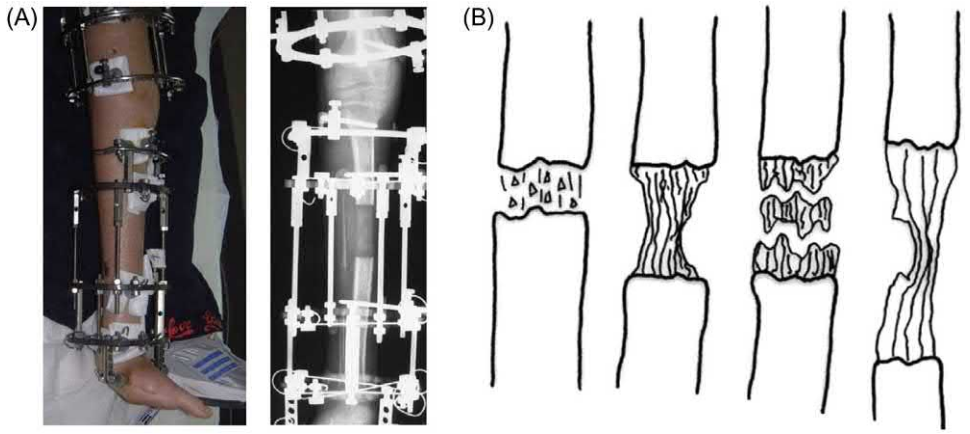

FIGURE 81.1 Bone regeneration using distraction osteogenesis.
(A) Typical distraction osteogenesis hardware (frame and rings) is shown. (B) Distraction osteogenesis proceeds in stages, the first of which is the *latency* stage during which the injury and repair process initiates and fracture callus forms, followed by the *distraction* stage in which the bone is gradually distracted through the osteotomy site at a rate of approximately 1 mm/day resulting in a fibrous interzone composed of mesenchymal progenitors. The interzone eventually revascularizes, and the cells differentiate and form regenerate bone through intramembranous and endochondral ossification. Finally, in the *consolidation* stage, the immature bone that formed in the defect is remodeled into lamellar bone. *Reproduced with permission from Catagni MA, Radwan M, Lovisetti L, Guerreschi F., Elmoghazy NA. Limb lengthening and deformity correction by the Ilizarov technique in type III fibular hemimelia: an alternative to amputation. Clin Orthop Relat Res 2011;469(4):1175–80.*

debridement following infections or atrophic nonunions. Previously, substantial bone defects have required amputation. In the 1960s an innovative technique for in vivo bone regeneration was developed in Russia by Ilizarov [42–44]. In the late 1980s this technique, referred to as distraction osteogenesis, was extended into Western Europe and the United States. Distraction osteogenesis is a process in which a bone is gradually separated at an osteotomy site. The bone on either end of the osteotomy is stabilized with an external fixation device (Fig. 81.1A). The regeneration of bone involves three stages [45]. The initial stage is termed latency and consists of a 5- to 10-day period during which the injury and repair process initiates and fracture callus forms. During the second, or distraction phase, the bone is gradually distracted through the osteotomy site at a rate of approximately 1 mm/day. Separation of the bone results in a repetitive tensile force in the distraction gap between the bone segments. In the center of the distraction a fibrous interzone composed of mesenchymal progenitors forms, while at each of the osteotomy surfaces bone the tissue is revascularized, and the cells differentiate and form regenerate bone through intramembranous and endochondral ossification. The final phase of the process is termed "consolidation" in which the immature bone formed in the defect is remodeled into lamellar bone (Fig. 81.1B).

Distraction osteogenesis has been used successfully to reconstruct limb defects resulting from tumor, trauma, infection, and developmental diseases [46–48]. The approach is also frequently used for the treatment of craniofacial defects [49]. The process of bone formation in distraction osteogenesis is dependent upon the mechanical stimulation of the healing bone callus that is generated by the tensile forces resulting from the daily separation of the bone fragments [45,50]. The tensile force results in the release of cytokines and growth factors that stimulate the proliferation and differentiation of mesenchymal stem cell progenitors and lead to angiogenesis and revascularization of the tissue. Growth factors associated with distraction osteogenesis include BMPs, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and Wnt signaling factors [51–53]. Thus distraction osteogenesis includes the various features associated with the successful engineering of regenerate bone: stabilization of the skeleton, mechanical stimulation, recruitment and stimulation of stem cell populations, angiogenesis, and bone incorporation and remodeling.

Despite these advantages, distraction osteogenesis is not an ideal approach to regenerate bone. While effective, distraction osteogenesis has a high complication rate. The stabilization of the bone with an external frame connected to pins or wires inserted into the bone is associated with pin tract infections in nearly all cases. Bone lengthening is also associated with a risk of joint contractures or stiffness, nerve palsy, and limb deformity [54]. Moreover, the entire process, and particularly the consolidation phase, requires an extensive period of time. In patients with war-related bone loss, fixation was maintained at approximately 1.5 months/cm of bone regeneration [46].

One area of need for bone tissue regeneration involves device-associated infections. These are typically treated with a two-stage reconstruction approach. This approach involves complete removal of the hardware and tissue debridement, and later reconstruction after eradication of the infection with antibiotics [55,56]. The use of poly (methyl methacrylate) or PMMA cement spacers impregnated with antibiotic agents in two-stage reconstruction has been common practice in Europe and has experienced widespread clinical use in US hospitals over the past few years [57,58]. These temporary spacers, which are typically installed after tissue debridement, are intended to provide sustained local elution of effective tissue concentrations of antibiotics to treat the infection while maintaining limb length, preserving tissue stock, minimizing soft tissue fibrosis and contraction, and in the case of articulating spacers, maintaining joint mobility until the lost tissue is reconstructed [59].

An innovative regenerative approach to treat this complicated scenario follows the two-stage guidelines of the Masquelet technique [60], which described the in situ formation of a periosteum-like pseudosynovial membrane around temporary antibiotic-eluting bone cement implants, which if preserved can provide nutrition and vascularization during the regenerative phase of the reconstruction. Fig. 81.2A shows an X-ray of a classic fractured tibia with atrophic nonunion and clinical evidence of osteomyelitis [6]. Stage 1 treatment of this case involved thorough debridement of the infected, necrotic bone and the placement of vancomycin-eluting PMMA cement beads in the resulting defect alongside the IM nail (Fig. 81.2B). This resulted in eradication of the infection and the endogenous formation of a pseudosynovial membrane over almost 1 year. The second stage then involved removal of antibiotic-eluting cement beads with careful preservation of the nutritive, induced biologic membrane, and subsequent installation of two titanium 15 mm diameter spinal cages to provide structural scaffolding for osteoinductive rhBMP-2 (INFUSE Bone Graft) that was placed "off-label" in the defect (Fig. 81.2C). To introduce BMP-2 responsive cells, autogenous cancellous bone and bone marrow were mixed with β-tricalcium sulfate granules (chronOS Bone Void Filler) and used to augment the defect, and the biological membrane was closed around the implanted biologics, morselized cells and tissue, and metallic scaffold. Four months following reconstruction, there was radiological evidence of new bone formation, and 1 year later there was resumption of independent ambulation with evidence of radiological and clinical healing [6] (Fig. 81.2D).

FIGURE 81.2 Tissue engineered reconstruction of a MRSA-infected tibial nonunion. (A) Radiograph of the MRSA-infected tibia 5 months after fracture, with no clinical evidence of healing. (B) Radiograph of the infected tibia after the first stage of the Masquelet technique, which involves resection of 17 cm of necrotic bone and insertion of vancomycin-eluting cement. (C) Radiograph following the second stage of the Masquelet technique, which involves exchanging the IM nail, with spinal cage support for INFUSE Bone Graft and autologous cancellous bone reamings. (D) Radiograph of the tibia 1 year following the two-stage Masquelet reconstruction, showing bone regeneration in the massive tibial defect. *IM*, Intermedullary; *MRSA*, methicillin-resistant *Staphylococcus aureus*. *Reproduced with permission from O'Malley NT, Kates SL. Advances on the Masquelet technique using a cage and nail construct. Arch Orthop Trauma Surg 2012;132(2):245–8.*

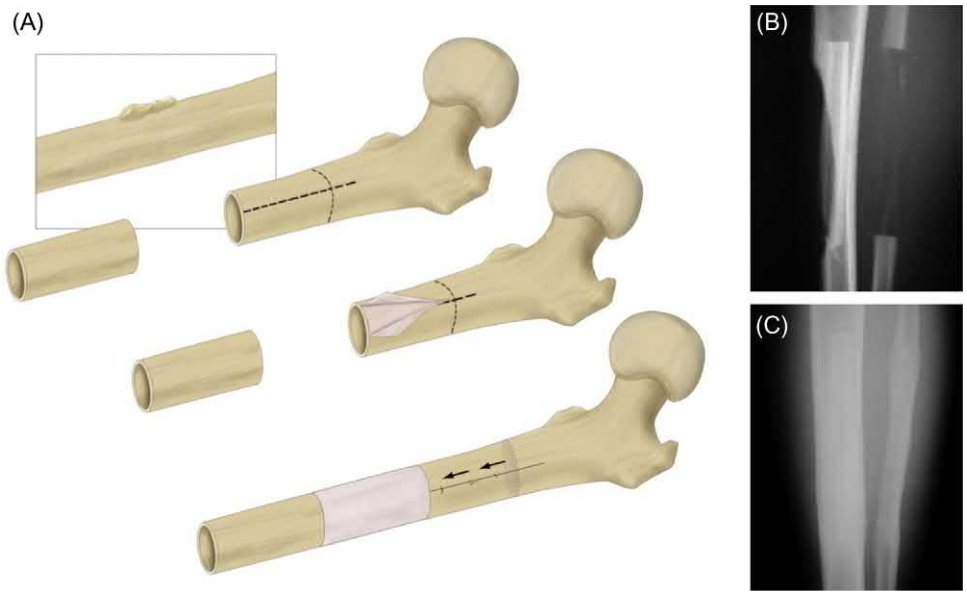

FIGURE 81.3 Exploiting the inherent regenerative capabilities of the periosteum to reconstruct the massive diaphyseal defects. (A) Schematic representation of the concept, which involves circumferential elevation a periosteal membrane off of healthy diaphyseal bone adjacent to the bone defect, and to then osteotomize the healthy bone (sans the periosteum) and transfer it to the defect out of the periosteal membrane. In theory the retained periosteum with its soft tissue attachment maintains vascularity and osteogenic progenitors. (B) Radiograph showing the reconstruction of a tibia with malignant cortical tumor with an autologous, pedicled fibula transfer, following elevation and in situ retention of the fibular periosteum. (C) Complete periosteum-enabled regeneration of the fibula is achieved by 3 months postreconstruction. *Reproduced from Knothe UR, Springfield DS. A novel surgical procedure for bridging of massive bone defects. World J Surg Oncol 2005;3(1):7, under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0).*

A similar approach to treat defects created by tumor resection exploits the endogenous osteoinductive properties of the periosteum and the osteoprogenitors it harbors. The concept is to circumferentially elevate a periosteal membrane off of healthy diaphyseal bone adjacent to the bone defect, and to then osteotomize the healthy bone (sans the periosteum) and transfer it to the defect out of the periosteal membrane. The retained periosteum with its soft tissue attachment maintains vascularity and osteogenesis can be augmented with cancellous bone grafts [61] (Fig. 81.3A). The feasibility of this approach was demonstrated in an osteosarcoma case by the reconstruction of

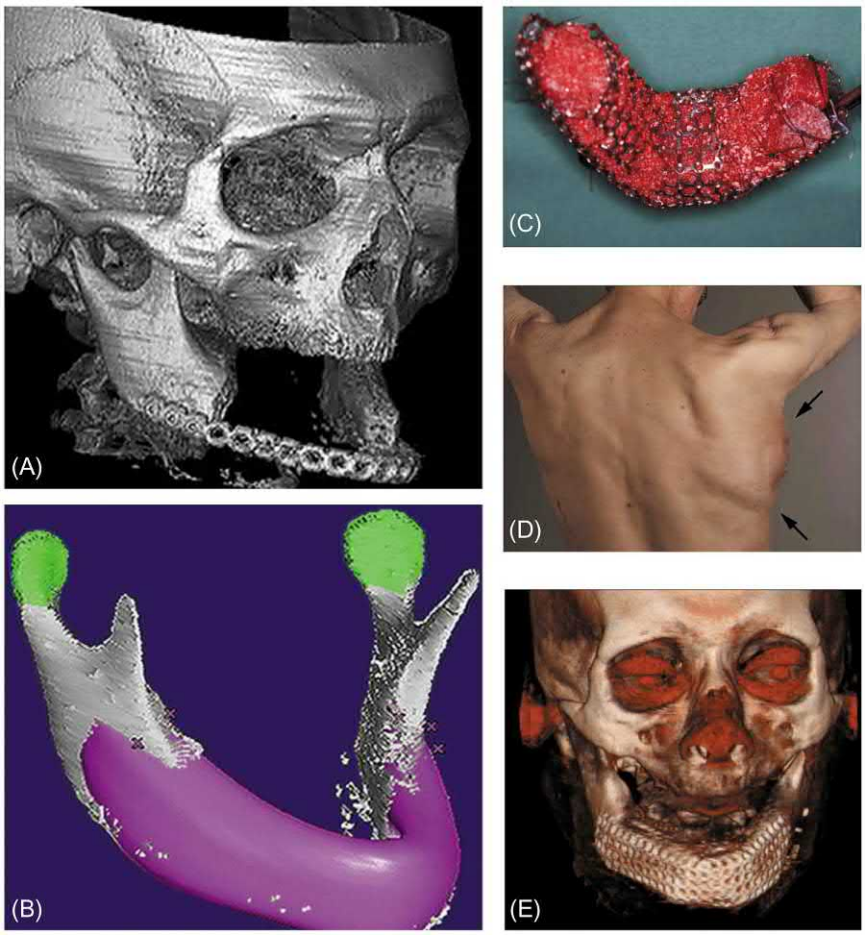

FIGURE 81.4 Image-guided tissue-engineered reconstruction of a massive mandibular defect. (A) The region of interest (jaw) is imaged using 3D CT. (B) The CT data is then fed to CAD software to generate an idealized virtual replacement of the missing parts of the mandible. (C) A titanium mesh is then formed in the shape of the missing bone model and augmented with BioOss hydroxyapatite blocks, OP-1 collagen implant, rhBMP-7, and autologous bone marrow aspirate. (D) The engineered mandibular graft is implanted in a heterotopic muscular pouch in the patient to establish vascularization and initial osteogenesis. (E) The graft was finally implanted orthotopically to reconstruct the mandibular defect. The patient had functional mastication and satisfactory esthetic outcome. *3D*, Three-dimensional; *CAD*, computer-aided design; *CT*, computed tomography. *Reproduced with permission from Warnke PH, Springer IN, Wiltfang J, Acil Y, Eufinger H, Wehmoller M, et al. Growth and transplantation of a custom vascularised bone graft in a man. Lancet 2004;364(9436):766–70.*

an iatrogenically created defect in the fibula following lifting and in situ retention of its periosteal membrane (Fig. 81.3B). The fibula graft was transferred to augment regeneration of a surface defect in the tibia that resulted from resection of a low-grade surface osteosarcoma. The periosteum membrane that was left behind was activated in situ to serve as an osteoinductive and osteoconductive sleeve and led to complete regeneration of the fibula defect within just 6 months (Fig. 81.3C).

The two approaches described previously utilize the principles of tissue stabilization, recruitment/delivery of stem cells, bone tissue differentiation, angiogenesis, and remodeling. The approaches also represent a personalized *point-of-care* method of tissue engineering of critical diaphyseal defects. They clearly borrow from the first principles of conventional tissue engineering, but circumvent the regulatory process via *off-label* use of several approved products (e.g., INFUSE, chronOS, and Bone Marrow). These cases represent individual success stories and demonstrate the safety and efficacy of biologics, scaffolds, and cells without in vitro manipulation; an alternative paradigm in tissue engineering that has been recognized for some time but one that has received less attention in the basic science literature. Advances in image-guided fabrication of biocompatible, osteoinductive scaffolds such as 3D bioprinting, which in theory enables simultaneous printing of biofactors and antibiotics within the patient-specific scaffold for sustained release can potentially lead to single-stage reconstruction procedures, accelerated recovery time, and improved the clinical outcome.

Clinical successes and opportunities in regenerative repair of craniofacial defects

The face distinguishes one human being from another. When the face is disfigured because of trauma, tumor removal, congenital anomalies, or chronic diseases, there is a critical need for functional and esthetic reconstruction [8]. These scenarios lend themselves to image-guided scaffold fabrication to restore the original shape of the face or jawbone. Success in this approach was first demonstrated by a report in the Lancet [9]. In this case the

patient, who lost a significant part of their mandible due to cancer, underwent 3D CT of the head and computer-aided design to generate an idealized virtual replacement of the missing parts of the mandible (Fig. 81.4A and B). A titanium mesh scaffold was then formed in the shape of the model and was filled with bone mineral blocks (BioOss Blocks) and augmented with bovine collagen type 1 (OP-1 implant) soaked with recombinant human BMP-7, and an autologous bone marrow aspirate to provide BMP-responsive osteo-regenerative cells (Fig. 81.4C). The titanium mesh cage was then implanted heterotopically into a pouch of the patient's right latissimus dorsi muscle (Fig. 81.4D) to establish vascularization and initial osteogenesis prior to orthotopic implantation to reconstruct the mandible (Fig. 81.4E). Skeletal scintigraphy bone scans showed evidence of uptake indicative of viable bone metabolism within the engineered mandible at both the heterotopic and orthotopic implantation sites, and postoperative CT imaging demonstrated radiographic evidence of healing and incorporation, with patient-reported functional improvements in chewing and a satisfactory esthetic outcome [9]. Other reports have since followed suit and demonstrated similar patient-specific, *point-of-care* reconstruction of the mandible with successful functional and esthetic outcomes [62,63].

Conclusion

Despite regulatory bottlenecks, surgeons and scientists will likely, and should, continue to push the frontiers of what is surgically possible, armed with innovations in cell-free tissue-engineering products, their skill and ingenuity, and most importantly their professional ethics and the vow they live by to do no harm. Cell-free refers to the recruitment of endogenous cells, including stem/progenitor cells, toward bone regeneration. Cell-free approaches arguably do not represent orthodox or conventional medical or tissue-engineering products from a regulatory or business standpoints, and the debates might not be settled by these $n = 1$ clinical reports, a far cry from randomized clinical trials required for level 1 data of safety and efficacy for regulatory approval. Nevertheless, these cases and similar ones not described herein successfully restored form, function, and quality of life to the afflicted patients. These *point-of-care* innovations might just be the bridge to wide clinical translatability and adoption of bone tissue-engineering approaches and products.

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Chapter 82

Tissue-engineered cardiovascular products

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Clinical situation/reality

Cardiovascular disease encompasses a number of clinical scenarios, including vascular disease, structural anomalies, (both congenital and acquired), and ischemic heart disease. In the United States, more than 800,000 people die from cardiovascular disease every year, that is, 1 in every 3 deaths according to the Centers for Disease Control and Prevention and US Department of Health and Human Services. The economic toll of cardiovascular disease is also massive, accounting for $350 billion in US health-care spending during 2014–15 [1].

The tissue engineering field employs biomaterials, cells, or their combination to develop biological substitutes that can restore, maintain, or improve tissue or organ function [2]. Since its inception, the intensive multidisciplinary nature of cardiovascular tissue engineering has revolutionized our understanding of cardiovascular biology and marshaled advances in both cell and scaffold technologies. The areas to which cardiovascular tissue engineering has mostly been applied are vascular and valve disease, congenital heart diseases, and more recently ischemic heart disease and heart failure. Only in two of these areas—valvular disease and congenital heart disease—have tissue engineering therapies (fixative-treated xenogeneic valves and patches made from pericardium) advanced to standard of care. However multiple tissue-engineered cardiovascular products are in clinical use or testing.

In this chapter, we will discuss these clinical conditions, the current state of the tissue engineering art and novel tissue engineering approaches.

Considerations for tissue-engineered cardiovascular constructs

The ultimate goal of tissue engineering is to produce a fully functional transplantable construct that cannot be distinguished from its original counterpart. Unfortunately, cardiovascular tissue engineering has not yet reached that pinnacle. Instead, cardiovascular tissue engineering is evolving from the use of simpler first-generation anatomically based products to more complex and function-based ones.

Most first-generation cardiovascular tissue engineering products were relatively simple nonliving scaffolds, which could be approved as medical devices. These included bioprostetic heart valves and fixative-treated xenogeneic pericardial membrane patches. Second-generation products, which have emerged more recently, are more complex and usually contain cells or other biologics impregnated on a synthetic or biologic scaffold. Due to their inherent complexity, these have to endure a much longer approval process involving phase 1–3 clinical trials to demonstrate safety and efficacy, unless they are able to obtain special classification (Fig. 82.1), which warrants a fast-track process. As the field matures and biomanufacturing becomes more real, the need for cardiovascular design standards has emerged. Given the complexity of cardiovascular diseases and the lack of anatomic redundancy, functional design considerations for each tissue-engineered cardiovascular product are complex. For a summary, see Tables 82.1 and 82.2.

Components for tissue-engineered cardiovascular constructs

Cell sources

Obtaining the quantities of cardiac cells of the right type needed for generation of living mature myocardium has been a significant bottleneck in the tissue engineering field. The human myocardium contains $\sim 10^{11}$ cells, of which one-third are cardiomyocytes. Terminally differentiated cardiomyocytes have a scant ability to proliferate

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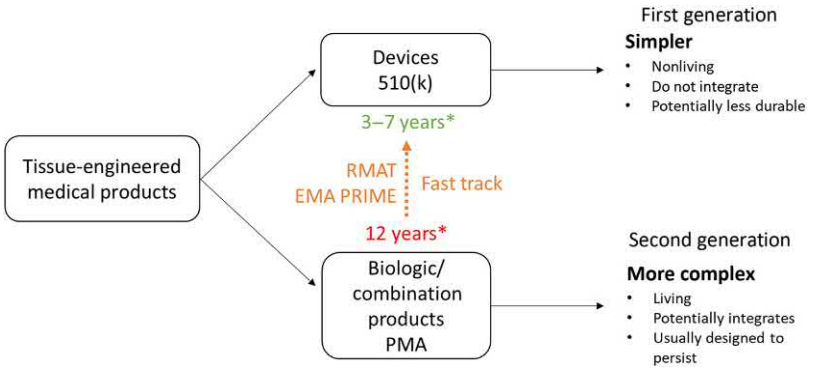

FIGURE 82.1 RMAT designation and EMA Prime can change the landscape of TEMPs approval. TEMPs categorized as devices are approved via the 510(k) pathway, which is up to 9 years shorter than the PMA pathway applied to biologics of combination products. RMAT and EMA Prime, via fast-track designation, could shorten the time frame for commercialization of PMA products. *EMA*, European Medicines Agency; *PMA*, premarket approval; *RMAT*, regenerative medicine advanced therapy; *TEMPs*, tissue-engineered Medical Product. * Timeline for approval taken from Ref. [3].

TABLE 82.1 **Food and Drug Administration (FDA)–licensed tissue-engineered medicine products.**

Proper name	Trade name	Manufacturer	STN
HPC, Cord Blood	ALLOCORD	SSM Cardinal Glennon Children's Medical Center	125413
HPC, Cord Blood	CLEVECORD	Cleveland Cord Blood Center	BL 125594
HPC, Cord Blood	DUCORD	Duke University School of Medicine	BL 125407
Allogeneic cultured keratinocytes and fibroblasts in bovine collagen	GINTUIT	Organogenesis Incorporated	BL 125400/0
HPC, Cord Blood	HEMACORD	New York Blood Center, Inc.	BL 125397
HPC, Cord Blood	–	Clinimmune Labs, University of Colorado Cord Blood Bank	BL 125391
HPC, Cord Blood	–	MD Anderson Cord Blood Bank	BL 125657
HPC, Cord Blood	–	LifeSouth Community Blood Centers, Inc.	BL 125432/0
HPC, Cord Blood		Bloodworks	125585
Talimogene laherparepvec	IMLYGIC	BioVex, Inc., a subsidiary of Amgen Inc.	125518
Tisagenlecleucel	KYMRIAH	Novartis Pharmaceuticals Corporation	125646
LAVIV	AZFICEL-T	Fibrocell Technologies	BL 125348/0
Voretigene neparvovec-rzyl	LUXTURNA	Spark Therapeutics, Inc.	125610
Autologous cultured chondrocytes on a porcine collagen membrane	MACI	Vericel Corp.	BL 125603
Sipuleucel-T	PROVENGE	Dendreon Corp.	BL 125197
Axicabtagene ciloleucel	YESCARTA	Kite Pharma, Incorporated	BL 125643
Cell sheet of human fibroblast in a shape of conduit, without further endothelialization	LIFELINE	Cytograft Tissue Engineering, Inc.	
Cell sheet of human fibroblast in a shape of conduit. ECs were seeded in the graft after devitalization of the luminal side	CYTOGRAFT	Cytograft Tissue Engineering, Inc.	
Decellularization of PGA scaffolds seeded with cadaver SMCs	HUMACYTE	Humacyte Incorporated, RTP, NC	
Decellularized bovine carotid artery graft	ARTEGRAFT	North Brunswick, NJ	
Onasemnogene abeparvovec-xioi	ZOLGENSMA	AveXis, Inc.	125694
Algisyl alginate	Algisyl-LVR	LoneStar Heart, Inc.	FDA 510(k) clearance
Porcine extracellular matrix	Cor PATCH	CorMatrix Cardiovascular, Inc.	FDA 510(k) clearance

STN, Submission tracking number.

TABLE 82.2 Clinical trials of cardiovascular tissue-engineered medicine products.

Study title	ClinicalTrials.gov identifier	Primary purpose	Study type model	Allocation	Status
First in Humans to Evaluate Collagen Patches With Stem Cells in Patients With Ischemic Left Ventricular Dysfunction (CARDIOMESH)	NCT03746938	Treatment	Interventional Single Group Assignment	Nonrandomized	Recruiting
Randomized Clinical Trial to Evaluate the Regenerative Capacity of CardioCell in Patients With Chronic Ischaemic Heart Failure (CIHF)	NCT03418233	Treatment	Interventional Parallel Assignment	Randomized	Recruiting
Transcoronary Infusion of Cardiac Progenitor Cells in Patients With Single Ventricle Physiology (TICAP)	NCT01273857	Treatment	Interventional Parallel Assignment	Nonrandomized	Completed
Randomized Study of Coronary Revascularization Surgery With Injection of WJ-MSCs and Placement of an Epicardial Extracellular Matrix (scorem-cells)	NCT04011059	Treatment	Interventional Parallel Assignment	Randomized	Not yet recruiting (new)
A Phase I, Open Label, Safety Study of INXN-4001 Delivered Via Retrograde Coronary Sinus Infusion in Patients With an Outpatient Left Ventricular Assist Device (LVAD)	NCT03409627	Treatment	Interventional Single Group Assignment	Nonrandomized	Recruiting
Human Umbilical Cord-Derived Mesenchymal Stem Cells With Injectable Collagen Scaffold Transplantation for Chronic Ischemic Cardiomyopathy	NCT02635464	Treatment	Interventional Parallel Assignment	Randomized	Active, not recruiting
Pericardic Adipose Pedicle Transposition Over the Myocardial Infarct (adiFLAP Trial) (adiFLAP)	NCT01473433	Treatment	Interventional Parallel Assignment	Randomized	Completed
CD133 + Autologous Cells After Myocardial Infarction	NCT00400959	Treatment	Interventional Parallel Assignment	Randomized	Completed
Randomized Controlled Pivotal Trial of Autologous Bone Marrow Mononuclear Cells Using the CardiAMP Cell Therapy System in Patients With Post Myocardial Infarction Heart Failure (CardiAMP Heart Failure Trial)	NCT02438306	Treatment	Interventional Parallel Assignment	Randomized	Recruiting
A Phase I, Open-Label Study of the Effects of Percutaneous Administration of an Extracellular Matrix Hydrogel, VentriGel, Following Myocardial Infarction	NCT02305602	Treatment	Interventional Single Group Assignment	Nonrandomized	Active, not recruiting
A Pilot Study Investigating the Clinical Use of Tissue Engineered Vascular Grafts in Congenital Heart Surgery	NCT01034007	Treatment	Interventional Single Group Assignment	Nonrandomized	Completed
Multi-center Assessment of Grafts in Coronaries: Long-term Evaluation of the C-Port Device	NCT01478061	Treatment	Interventional Single Group Assignment	Nonrandomized	Completed
Safety and Efficacy of a Vascular Prosthesis for Hemodialysis Access in Patients With End-Stage Renal Disease	NCT01840956	Treatment	Interventional Single Group Assignment	Nonrandomized	Completed
A Pilot Study for Evaluation of the Safety and Efficacy of Humacyte's Human Acellular Vascular Graft for Use as a Vascular Prosthesis for Hemodialysis Access in Patients With End-Stage Renal Disease	NCT01744418	Treatment	Interventional Single Group Assignment	Nonrandomized	Active, not recruiting
Phase I Open-label, First-in-human Study to Evaluate Feasibility and Safety of Tissue Engineered Veins in Patients With Chronic Venous Insufficiency	NCT03784131	Treatment	Interventional Single Group Assignment	Nonrandomized	Not yet recruiting

and therefore cannot be expanded in culture for most tissue engineering applications. Thus many studies have examined the efficacy of seeding scaffolds with a variety of cardiac stem and progenitor cell populations [4,5] rather than with mature cardiomyocytes. Although this provides "enough" cells, obtaining mature function from these immature cells remains a hurdle.

Considering the highly limited proliferative nature of cardiomyocytes, the large-scale differentiation of cardiomyocytes from human-induced pluripotent stem cells (hiPSCs) has revolutionized cardiac tissue engineering. hiPSCs remain the most feasible source of cells for generation of an autologous tissue or organ because they can be generated from patients of any age and can be expanded to large numbers in vitro [6,7]. If the goal is to seed a scaffold prior to implantation, a large number of cells—in the millions and billions, depending on tissue volume—must be generated and maintained in vitro. For this, several efficient differentiation protocols have been reported, but reliable scale up to billions of mature cardiomyocytes remains a challenge using existing technologies. [8] Nonetheless, other cell sources are being utilized for both tissue engineering and regenerative medicine applications, including bone marrow− or tissue-derived endothelial cells and mesenchymal stem cells [9−11]. Cells are a critical tissue engineering biomaterial, but cells alone do not comprise a tissue or an organ.

Scaffolds

Cells are a critical tissue engineering biomaterial, but cells alone do not comprise a tissue or an organ. Cells are often seeded onto scaffolds of biologic or synthetic origin. However, scaffolds can be used with or without cells in tissue engineering products. Scaffold sources are reviewed in-depth elsewhere [12].

Synthetic scaffolds

Hydrogels are simplified hydrophilic polymer networks obtained from both natural and synthetic sources that have been used as scaffolds in both the cardiac and vascular fields [13,14]. Some of the widely used hydrogels include collagen [15,16], gelatin [17], Matrigel [18], alginate [19], fibrin [20], poly(2-hydroxyethyl methacrylate) [21], poly(*N*-isopropyl acrylamide) [22], and poly(ethylene glycol) (PEG) [23]. Hydrogels provide mechanical support for cells to deposit extracellular matrix (ECM) and form newly synthesized tissues as they degrade [23,24]. Furthermore, the physical and chemical properties of hydrogels can be tuned to enhance cell viability and function. These materials can be utilized either by themselves or in combination with cells. In particular, hydrogel-based materials have been successfully utilized in cardiac tissue engineering as structural/mechanical supports in failing heart, as cell delivery tools to increase cell retention in vivo, and as growth factor delivery agents to promote vascularization after cell transplantation. Yet, despite the fact that the effect of hydrogel on long-term cell survival has been positive [25−27], the injection pressure necessary for in vivo cell administration in the presence of hydrogel is high, which can cause extensive early cell death and ultimately decrease cell delivery.

Although hydrogels are often viewed as simplified ECM, they have several disadvantages that impact their use in tissue engineering. First, controlling the physicochemical properties of hydrogels composed of natural materials is difficult, which can lead to degradation of these substances after implantation, and can pose challenges during their purification and sterilization [28]. Consequently, synthetic hydrogels such as PEG, polylactic acid, polylactic-*co*-glycolic acid, polycaprolactone, polyacrylamide, and polyurethane have been developed to minimize these drawbacks [29]. However, the cytotoxic potential of synthetic hydrogels is still under study, and the Food and Drug Administration (FDA) has approved only the use of PEG, polylactic acid, and polylactic-*co*-glycolic acid for clinical application. In addition, PEG is sensitive to matrix metalloproteinases, which are found in heart and modulate its elasticity altering biophysical and biochemical parameters that in turn could influence (cardiomyogenic) differentiation of any implanted cells [30]. Because of these disadvantages, the use of natural scaffolds, such as decellularized ECM (dECM), has been highlighted as a more promising tool for repair strategies.

Extracellular matrix as scaffold

Since the early days of cardiac tissue engineering, ECM proteins have been used to functionalize synthetic scaffolds to provide a native-like extracellular microenvironment. Traditionally, bioartificial scaffolds were created using ECM components and different types of commercially available natural hydrogels such as Matrigel (laminin, collagen type IV, and heparan sulfate) [25,26], collagen [27], or fibrin [31].

The regenerative medicine field has increasingly recognized the central role of ECM in the success of tissue and organ engineering applications. During development, cells coalesce with ECM forming functional units unique to each organ. ECM is composed mainly of different types of collagen, glycosaminoglycans, fibronectin, laminin, elastin, and growth factors. The highly organized spatial anisotropy of ECM provides unique biochemical cues and architecture, which are difficult to mimic. Degradation of many of these native compounds results in the release of soluble peptide molecules involved in regulating many biological functions of a tissue organ, such as

cell proliferation, migration, and differentiation, and angiogenesis [32–34].

ECM can be used as a tissue engineering scaffold in multiple physical forms: as a reconstituted powder, hydrogel, or in its original form as a macrostructure. Our group has focused on ECM as a scaffold, especially dECM. Utilizing a variety of detergents the decellularization process removes cellular content, leaving behind an elegant 3D structure with unique tissue–organ-specific composition and architecture. Both the composition and the 3D spatial organization of dECM bioartificial scaffolds are fundamental for promoting cell growth and differentiation, although it is unclear which of the two factors is more critical [35–39].

Decellularized extracellular matrix as an ideal scaffold

To be used as a scaffold or in vivo, ECM must be decellularized because cells elicit a major antigenic response [40,41]. The ideal decellularization protocol results in the elimination of allogeneic and xenogeneic antigens as well as the cellular and nuclear content of the tissue but preserves the composition, physiological properties, mechanical integrity, and vascular structure of the ECM.

Investigators have developed a wide spectrum of techniques to exploit the advantages of ECM in cardiovascular tissue engineering. Today, dECM is used alone or mixed with other biomaterials both in vitro and in vivo along the continuum from bench to bedside as an injectable therapy for heart failure [42]. dECM scaffolds colonized with cells of both cardiomyogenic and endothelial lineages prior to implantation were reported to be successfully remodeled in different animal models [43–46]. Implantation of dECM of myocardial origin into the right ventricle has been reported to restore regional mechanical function in preclinical models of ischemic heart failure [47,48], and in humans [49]. Numerous studies have reported that implantation of dECM facilitates the remodeling of different types of tissues, both in animal models and in the human clinical setting [50–57]. There is growing evidence of the capability of dECM to polarize the immune response toward a more regenerative path by direct injection of dECM in various forms [58].

Perfusion decellularization

The organ engineering field was transformed in 2008 with our group's publication of a method of whole organ perfusion decellularization and partial recellularization with neonatal rat cardiac cells [59]. By perfusing the organ vasculature with decellularization agents, cadaveric organs were stripped of their resident cell components. [40,60,61] The resulting scaffold contained a holistic ECM, including all compartments of the organ. It preserved the 3D composition and biologic activity of ECM, making it an attractive material for cell adhesion, differentiation, and proliferation [50,54,56]. This advance has enabled labs worldwide to generate organ and scaffolds with the complex functionality of native organ, including myocardium for tissue and whole organ engineering applications.

Tissue-engineered cardiovascular constructs

Vascular grafts

Coronary heart disease and peripheral vascular disease have a combined annual mortality incidence predicted to rise to 23.3 million worldwide by 2030. Current standard of care includes invasive approaches to reestablish flow through damaged vessels, ranging from percutaneous dilation of the vessel at the occlusion site by angioplasty or stent placement, to surgical bypass grafting. Autologous saphenous veins were first used as a vascular graft in a clinical application in the early 1950s. Today, autologous vessels (saphenous vein and internal thoracic artery) are the gold-standard grafts for small-diameter vessels; however, their use requires invasive harvesting and is limited to providing four grafts, which could be a problem for some patients.

An alternative to autologous vessels is the use of synthetic vascular grafts such as Dacron [62] and PTFE [63]. Synthetic grafts were first used in the 1970s for aortic and lower extremity bypass, respectively. Today, they are standard of care for large-diameter vessels. However, thrombosis is the leading cause of failure when synthetic grafts are used to bypass small-diameter vessels (coronary arteries and arteries below the knee).

Biologically based vascular constructs were developed to overcome the limitations of synthetic vascular grafts. Since the late 1970s acellular vascular grafts from bovine and human origin [64,65] (Artegraft, North Brunswick, NJ), Cryovein (CryoLife, Kennesaw, GA) have been commercially available for use primarily in small vessels. In the 1980s Weinberg and Bell first tried to fabricate biological grafts containing xenogeneic cells, including fibroblasts, vascular smooth muscle cells, and endothelial cells, embedded in collagen [66]. Although these rudimentary grafts recreated vessel layers (adventitia, media, and intima), they had poor mechanical properties and required a Dacron mesh to act as a structural support.

Today, two main approaches exist for generating autologous tissue-engineered vascular grafts (TEVGs): in vitro seeding of a mix of autologous cells (endothelial, smooth muscle cells, and fibroblasts) onto decellularized scaffolds, and in vivo recellularization of decellularized scaffolds by harnessing endogenous processes. Ideally,

TEVGs would be capable of growing and remodeling in vivo [67], have antithrombogenic properties and no immunogenic activity, and be similar mechanically to the corresponding native tissue. TEVGs are poised to be a potent therapeutic tool in vascular replacement.

Scaffold-free grafts

Tissue-engineered self-assembly is a technology for creating TEVGs based on cells alone—without a scaffold. Cell-sheet TEVGs are the most clinically advanced technique for generating scaffold-free TEVGs. The technique emerged in 1998 after the first clinical trial results were published [68]. Cell-sheet TEVG involves the use of a dense and cohesive sheet of cells rolled around a mandrel and matured under dynamic conditions to create a vessel-like construct. Since the first successful report [68], several the technique has been optimize using different cell types, including human adipose−derived stromal cells, dermal fibroblasts, vascular smooth muscle cells, mesenchymal stromal cells, and endothelial cells [69−75].

Novel tissue engineering self-assembly techniques in the preclinical phase include proof-of-concept vascular structures created by fusion of cell spheroids to form a tubular structure [76], or bioprinted vascular tree [77]. While these new cell-based approaches are promising, to date their use is limited due to a lack of mechanical and structural stability.

Scaffold-based tissue-engineered vascular grafts

Strictly speaking, both synthetic vascular grafts and acellular vessels are scaffold-based TEVGs. Scaffold support provides a physiological template on which cells can attach, proliferate, and integrate; and depending on the source, it may provide biological cues related to its composition and micro/macro-architecture. Currently, a more sophisticated TEVG with autologous cells is an unmet need being pursued by multiple groups [67,78], by using innovative tools to enhance cell seeding, proliferation, and maturation. To date, decellularized human vessel constructs are the most successful TEVGs when applied in vivo, although postimplantation thrombus events remain the major limitation to long-term patency [79].

There is currently a well-known, critical unmet need for novel vascular graft replacements for congenital heart disease. Existing synthetic vascular grafts are unable to grow and mimic the mechanical properties of the native tissue. Without potential for growth and remodeling in the native tissue, graft material−related failures are the leading cause of morbidity and mortality in the pediatric population. For patients born with single ventricle disease, the current standard of care includes surgical deviation of blood flow (e.g., Fontan procedure) using grafts, mostly of synthetic origin. Generating off-the-shelf TEVGs made from allogeneic [80] or autologous [81] cells for use in the Fontan circulation is an area of active research. In 2018 a phase 1 clinical study of four patients was completed evaluating the safety of TEVG as an extracardiac total cavopulmonary connection in single ventricle disease (NCT:01034007).

Another area where TEVGs are likely to have a major impact is in the treatment of peripheral artery disease. As previously stated, thrombogenic failure of synthetic grafts increases as vessel diameter decreases. New solutions are needed for medium- and small-sized vessels requiring bypass. As of August 2019, when using the terms “peripheral arterial disease” and “tissue graft,” only two clinical studies appear: one using synthetic scaffold coated with human allogeneic cells, and one using a novel acellular human vascular prosthesis. Both trials are active but not yet recruiting.

In summary, vascular grafts have been and will continue to be a major area of interest for tissue engineering, but in 2019 the field has not progressed to the point that functional vessels—at least in small-diameter vessels—are a clinical reality.

Valves

Every year, more than 5 million people in the United States are diagnosed with valvular heart disease [82]. No effective medical treatment exists to halt chronic valve dysfunction or disease progression. In 2010 approximately 106,000 valve replacements were performed in the United States [83] and the number is expected to increase to 850,000 by 2050.

Current valve prostheses

The standard of care for medically untreatable heart valve disease is surgical intervention. When repair is not feasible, replacement of the diseased valve with a device—a mechanical or biological prosthetic valve—each with benefits and drawbacks, is then mandatory.

Mechanical valves are considered more durable than biologic valves, usually lasting throughout a patient’s life span. However, mechanical valves carry a high risk of thromboembolism and require lifelong anticoagulation therapy. Bioprosthetic valves provide better hemodynamics and do not require long-term anticoagulation therapy; but, because of their biologic composition, they are prone to deterioration and complications resulting from immune reactions [84]. Thus current bioprosthetic valves have a limited life span, usually failing due to rupture or calcification. Neither mechanical nor biologic prosthetic valves have growth capability and therefore neither is ideal in pediatric patients. Next-generation tissue-engineered constructs are being developed to overcome these limitations.

Tissue-engineered valves

The ideal tissue-engineered heart valve would be a living conduit that could adapt and grow with the patient. Theoretically, a tissue-engineered valve would have laminar flow, eliminating the need for anticoagulation therapy; could actively remodel, preventing degeneration; and would grow as needed—all cumulatively preventing the need for reoperation. These valves would have the potential to overcome the limitations of existing prosthetics, which would improve the safety and outcomes of valve replacement procedures.

Although basic tissue engineering concepts have been applied to generate valves from cadaveric tissues for decades, these prostheses are most commonly built from porcine or bovine sources using acellular fixative-treated valvular constituents that the body recognizes as immunogenic. Furthermore, most first-generation valve constructs are mounted on nonbiological struts to hold the tissue at a desired anatomical conformation. Cell-containing cryopreserved human valve allografts are a xeno-free alternative made from human cadaveric tissue after valve fixation. Clinical experience has shown that these allografts fail within a decade [85], most likely when an immunological response leads to deterioration of the valve. Decellularization, or use of detergents and other chemicals to remove resident cell components, has been employed to reduce the immunogenicity of these allografts, but clinical trial results have been mixed [86,87]. Matrix-P and Matrix-P Plus were the first nonfixed decellularized xenogeneic valve substitutes tested for clinical use. These commercially available valve replacements are recellularized with autologous cells. Follow-up of patients implanted with Matrix-P valves showed very low graft failure in humans ($n = 3$ vs $n = 15 + n = 14$ uneventful), but other investigators reported poor effective viability in preclinical settings and poor recruitment of host cells, which was interpreted as a risk for graft dysfunction [88,89].

A newer approach is to repopulate decellularized valve scaffolds with autologous or xenogeneic cells prior to implantation. However, preclinical studies of implanted recellularized valves have shown moderate to limited success [90], which could be related to the inability of phenotypically appropriate cells to recellularize the valve leaflets. Currently, scientists are working on determining optimal recellularization methods, such as in vitro recellularization combined with chemical and/or mechanical conditioning [90]. In situ recellularization strategies have been described in studies of decellularized homografts, where the recruitment of endothelial and interstitial cells for valve and cavities occurs in vivo [91].

In summary, prosthetic valve replacement is an established, lifesaving therapy for patients with untreatable heart valve disease, although there are clear drawbacks to existing mechanical and biologic prostheses. Advances in tissue engineering and regenerative medicine have made it possible to envision and design a tissue-engineered valve that can function as a true replacement—a valve that grows with the patient, adapts to the microenvironment, and lasts the duration of the patient's life span. Optimizing recellularization techniques would allow for repopulation of decellularized valves with autologous cells, reducing risks of immunogenicity and thromboembolism.

Cardiac patches

Myocardial infarction, known colloquially as a heart attack, is the most common cause for the loss of large numbers of cardiomyocytes and therefore a decrease of the pumping power of the heart. During a heart attack, oxygen-rich blood flow in the culprit vessel decreases or ceases; if blood flow is not restored quickly, heart muscle cells in the downstream territory to die and are replaced by scar tissue. In the heart, scar tissue is an electromechanically inefficient area that fails to conduct or contract appropriately. In addition, inflammation occurs that drives a remodeling process that affects surrounding viable areas.

Cell therapy is a related field to tissue engineering that for the past two decades and has been trying to fulfill the promise to repair the damaged heart. Multiple cell types have been utilized, including mesenchymal stromal cells, adipose-derived stem cells and cardiopoietic progenitor, or pluripotent stem cells. Nonetheless, outcomes in the clinical use of cells have been disappointing. Beneficial clinical effects are frequently observed but are not often statistically significant [6,7,92,93]. In 2016 the European Society of Cardiology Working Group—Cell Biology of the Heart concluded, "The early promise of cell therapy has not yet been fulfilled"[7].

Cell therapy is premised on the concept of delivering a large number of cells to the site of injury that can subsequently influence the formation and maturation of scar or promote endogenous repair. However, studies have shown that within 96 hours of local cell injection, less than 25% of delivered cells are retained at the site of injection, or within the myocardium—severely limiting the local efficacy of cell-based cardiovascular treatments [94,95]. Improving retention of cells or cell secretome at the injury site could provide a major benefit to cell delivery. Researchers have been investigating the benefits of using cardiac patches or gels to deliver cells to increase cell retention.

Noncontractile cardiac patches

Cardiac tissue engineering approaches aim to prevent myocardial scarring, to slow or halt scar expansion, and/

or to restore the scar tissue to a state of working muscle that can electromechanically reintegrate into functional myocardium. Regenerative medicine approaches to prevent or repair myocardial scarring comprise simply delivering cells at the time of injury, or shortly thereafter, to mitigate scar formation/expansion and using scaffolds containing cells or other biologics to manipulate the damaged microenvironment.

A promising tissue engineering approach is using a scaffold to deliver cells directly on the epicardial surface of the injury site to cover and overlap the damaged region [6,7]. Studies have shown that ECM-based patches can serve as scaffolds that provide active support of cardiac muscle as well as biochemical signaling via the direct contact of the biomaterials that deliver paracrine signaling from incorporated cells.

Other applications of cardiac patches include repair of congenital or acquired damage to heart anatomical structures. Currently, patches produced from membranes, such as decellularized pericardium, are engineered from xenogeneic or autologous sources after being fixed and preserved [96]. Although these patches are generated based on basic tissue engineering principles, they are unable to grow with the organ and do not contain the histological and mechanical properties of the structure they are supposed to replace.

Bioprinted patches

The 3D printing or additive manufacturing of cardiac patches entails the computer-controlled layer-by-layer deposition of natural or synthetic polymers and living cells. The ability to control the internal shape and microarchitecture is a major advantage of this technique. Although bioprinting may eventually allow for the precise spatial control needed to fabricate integrative 3D structures [97], this approach currently faces many obstacles, especially related to the inability to scale up without low cellularity, the inability to generate vasculature, and the failure to obtain mature cells [98]. We believe that utilizing native ECM as a cross-linked bioink is currently the most effective option to address the required complexity and the most straightforward solution to many of the 3D bioprinting hurdles.

Contractile patches

Contracting integrated cardiac patches would be a tissue-engineered homerun. However, at present most contractile patches are epicardially applied constructs made of ECM or ECM components (e.g., collagen) impregnated with cells, using the engineered heart tissue (EHT) approach. Early EHT technology resulted in the vitro development of a contractile collagen ring–based patch that could be implanted epicardially. Using in vitro generation and bioreactor-assisted cultivation, the EHT approach moved from proof-of-concept in the late 1990s to the contemporary EHT constructs using iPSC technology [99,100].

Although these patches are contractile, EHT constructs lack vasculature and are limited in size and depth. Our approach to generating contractile patches is unique. We recellularize whole heart dECM with human endothelial cells and cardiomyocytes, mature it in a 3D bioreactor, and then excise a ventricular patch—generating a construct with micro- and macro-characteristics very similar the native tissue [101].

Hurdles for using cardiac patches

Achieving vascularization—a must for a beating patch A holy grail of cardiac tissue engineering is effective vascularization within a cardiac tissue scaffold to allow a continuous diffusion of nutrients and oxygen to the cells even in the interior of a thick construct. Several approaches have been developed to introduce vascular structures in scaffolds, mainly based on the delivery and stimulation of endothelial cells in complex hydrogel systems [102–105].

Yet, at the current state of research and development in tissue-engineered cardiac patches, we believe that the elegant simplicity of the methodology—casting cells and avoiding complicated seeding techniques—is likely to be undermined by the complexity required to provide sufficient vascularization for transit of nutrients and metabolites sufficient to permit cell survival. In the short term, utilizing decellularized ventricular wall patches that provide both native thickness and a complex vascular architecture is recommended [15]. Given the current ability to reendothelialize the vascular tree in both small and large hearts [15], we have developed a staged recellularization method that permits perfusable constructs.

Successful vascularization may promote the migration and incorporation of cells inside the damaged myocardium. Here, the role of ECM in achieving vascularization of a cardiac tissue-engineered construct. In decellularized organ scaffolds the ECM serves the dual purpose of providing structural support material and serving as a medium for the exchange of biochemical signals with the cells that adhere to it [106,107]. The ECM is also responsible for maintaining an adequate network of blood vessels and for binding growth factors, which preserve the appropriate structure of the organ during development and repair [108]. These characteristics are especially challenging to mimic when generating complex tissues or organs with heterogeneous cellular components.

Achieving electromechanical integration To achieve the goal of electromechanical integration and support, the mechanisms and techniques for driving cardiomyocyte

maturation are being widely investigated, and transferring these methods to generate clinically relevant cells is a focus of topical research [109–111]. Bioreactors are essential tools needed to translate these concepts into appropriate methods and to guide the necessary bioprocesses. In vitro studies designed to mimic the cardiac niche showed that biophysical stimulation by uniaxial stretching fundamentally affected the maturation of myocardial patches with various cell types and ECM compositions [112–115]. Another well-established key concept of biophysical stimulation is electrical stimulation by fields or contacting electrodes [116–118]. Also, the combined effects of both mechanical and electrical stimulation or the supremacy of one method are under debate and investigation [113,119–121]. Significant effort has been invested in optimizing bioreactor design according to good manufacturing practice requirements to enable a broad spectrum of tissue engineering applications [122]. These efforts must address the demand for a sufficient level of maturity to enable electromechanical integration; however, the technology is not currently available to replicate the highly complex, spatially vectored design of the native cardiac tissue in the architecture of artificially designed scaffolds.

A large-scale functional patch cannot achieve electromechanical integration into the native host myocardium based on the maturity of cardiomyocytes alone. The spatial organization of the cells must match with the high anisotropic structure that is regionally specific to the superordinate framework of the left ventricle. In other words, the patch needs to precisely fit the specific region of injury whose architecture varies based on individual complex heart topography.

Building the next level of complexity: whole heart

Although early repair is a goal of cardiovascular tissue engineering, the fact remains that heart transplantation is the only effective treatment for end-stage heart failure, and that the unmet need for donor organs across the globe is increasing unabated. While cardiac cellular reprograming and endogenous repair strategies have raised excitement and 3D printing has captivated the world, these approaches are in their infancy and have generated results unlikely to be translatable to humans for decades. Tissue-engineered cardiac patches may be promising for cardiac repair but are unlikely to address the worldwide organ shortage. Whole organ engineering has the potential to alleviate this by providing an alternative source of organ replacements from animal and human sources.

Generating functional hearts of any significant size is daunting. But the field of whole organ tissue engineering was transformed in 2008 when the publication of whole heart perfusion decellularization occurred. The ability to generate whole organ scaffolds with an intact but acellular vascular network provided the first opportunity to build cardiac tissue of more than a few cells thick. Engineering a bioartificial heart like any other tissue-engineered construct requires scaffolds, (human) cells, bioreactors, and controllable processes.

The number of published tissue-engineered whole heart studies worldwide is remarkably low [59,118,123–131]. The paucity of whole heart research could be attributed to the work involved in creating a single whole heart construct that overlaps several areas in tissue engineering and regenerative medicine. Furthermore, whole heart engineering entails extensive capital resources and intensive time and labor. Perhaps the more important issue is that whole heart engineering demands coordinated, high level of expertise, experience, and technology from multiple scientific disciplines, including cellular biology, mechanical and chemical engineering, cardiac electrophysiology, and cardiac surgery. This high level of multidisciplinary expertise is needed to overcome numerous technical and scientific hurdles: nondestructive decellularization techniques, scaling up and out of cell manufacturing, efficient cell delivery, and efficient whole organ repopulation strategies. Moreover, bioprocessing strategies and bioreactor technology are needed to provide controllable growth and maturation at the level of an organ.

Researchers in the whole heart engineering field have overcome many of these technical and component hurdles to generate decellularized whole heart constructs repopulated with hiPSCs. Lu et al. demonstrated that hiPSC-derived cardiomyocytes infused into the vasculature of a small rodent decellularized heart survived and contracted the wall [128]. Guyette et al. showed that injection of hiPSC-derived cardiomyocytes in human whole heart scaffolds resulted in contractile ventricular muscle [124]. Recently, porcine whole heart scaffolds were shown to recruit cells for remuscularization in a xenogeneic environment [130]. Despite progress toward a replacement bioartificial heart, effective recellularization is currently a major hurdle in the field.

Now, after a decade of whole organ engineering research and innovation, whole heart tissue engineering is an established field. Building a bioartificial heart remains an achievable end goal of cardiovascular bioengineering within our lifetime [132]. The current challenge is to improve and scale up/out the processes required for successful, full recellularization of a whole heart scaffold. This will entail generating sufficient numbers of high-quality cardiac cells, fully repopulating a scaffold with the cells, and maturing a physiologically functional recellularized human-sized heart. Maintaining sterility and

quantifying readiness of the nascent organ will also be critical for success [98].

Pathway to approval and commercialization

In the relatively young field of tissue—and especially whole organ engineering—there are no established standard methods to be followed. Methodology used to produce scaffolds, repopulate them with cells, engineer and produce cells, and to process engineered tissues lacks defined parameters. However, bioanalytical tools to develop critical process parameter and critical quality attributes for construct evaluation are the subject of ongoing research and scientific discussion. Furthermore, as tissue-engineered myocardial constructs move from simpler first-generation nonliving cardiac patches to more complex biologics, combination products or even organs, increasing discussion will need to occur about the best models for preclinical use and the design of first in human studies [101,121,133,134].

Biomanufacturing of tissues and organs will need to be automated if the technology is to be scaled to meet the global cardiovascular disease burden. Generally, the production of tissue-engineered constructs can be described as a sequential progression of scaffold preparation, repopulation, and bioreactor-assisted processing, of the resulting construct based on perfusion systems. However, for every single step, there is still a lack of nondestructive quality control and process automation that precludes the commercial development of these constructs on an industrial scale. Process analytics, development of standards, and controlled methodology will be the key to moving forward [125,135–138] (Fig. 82.2). To this end, over the past decade multiple organizations have emerged that are dedicated to the development of tissue engineering standards, and biomanufacturing processes. A brief compilation of these standard generating organizations along with governmental agencies that impact product approval in Europe and the United States are collected in Table 82.3.

To give patients access to these new regenerative medicine technologies, both the European Union and the US regulatory agencies have pioneered specific programs to expedite the pathway to approval. The European Medicines Agency (EMA) launched the PRIME (priority medicines) scheme on the basis of enhanced interaction and early dialog with developers of promising medicines that target an unmet medical need, to optimize development plans and speed up evaluation so these medicines can reach patients earlier. One of the main goals is to help the developers improve the design of clinical trials so that the data generated is suitable for evaluating a marketing authorization application (https://www.ema.europa.eu/en/human-regulatory/research-development/prime-priority-medicines). Similarly, the US FDA was authorized in Section 3033 of *the 21st Century Cures Act* to provide "regenerative medicine advanced therapy (RMAT) designation" to biologic therapies including tissue-engineered products that "are intended to treat, modify, reverse or

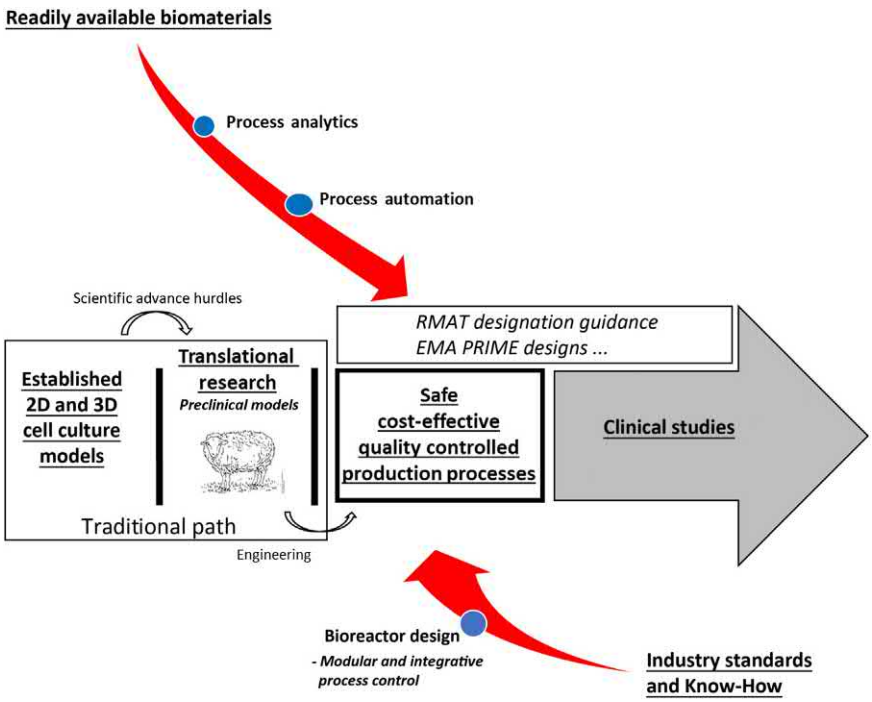

FIGURE 82.2 Steps for moving biomanufacturing of organs and tissues from bench to clinical use. Traditionally, candidate products established on in vitro models need to be tested in preclinical studies prior to the initiation of clinical trials. Scaling up biomanufacturing of tissue-engineered medical products will require developing process parameters that enable the use of readily available biomaterials, based on industry standards and know-how that yield safe processes for their use in the approval pathway. *EMA*, European Medicines Agency; *RMAT*, regenerative medicine advanced therapy.

TABLE 82.3 Standard development bodies in tissue engineering.

Agency	Goal	Website
ASTM International, *formerly known as American Society for Testing and Materials (ASTME)*	ASTM integrates consensus standards—developed with its international membership of volunteer technical experts—and innovative services to improve lives	www.astm.org
The National Institute of Standards and Technology (NIST)	NIST laboratory programs serve the cellular and gene therapy and regenerative medicine community through the development of a measurement infrastructure, including enabling tools, methods and protocols, bioinformatics and modeling tools as well as documentary standards and reference materials	https://www.nist.gov/ https://www.standardscoordinatingbody.org/
The Standards Coordinating Body for Gene, Cell and Regenerative Medicines and Cell-based Drug Discovery (SCB)	Launched by the ARM in 2017, the SCB's mission is to coordinate the accelerated advancement and improved awareness of the standards and best practices that address the rapidly evolving needs of the global regenerative medicine advanced therapyThe SCB and the NIST are jointly coordinating and contributing to the development of standards for accelerating R&D and clinical translation of regenerative medicine and advanced therapies	
The National Institute for Innovation in Manufacturing Biopharmaceuticals (NIIMBL)	NIIMBL is a public−private partnership dedicated to advancing biopharmaceutical manufacturing innovation and workforce development. Together with partners from industry, academia, nonprofits, and government agencies, NIIMBL collaborates on innovative manufacturing technologies that bring these lifesaving and life-enhancing products to market faster and at reduced cost, while maintaining safety and efficacy. In conjunction with technology advancement, it works to establish reference standards and measurement technologies to enhance efficiencies in the manufacturing process. Finally, NIIMBL also strives to cultivate a world-leading biopharmaceutical workforce through novel training and education programs	https://niimbl.force.com/s/
Institute of Electrical and Electronics Engineers (IEEE) Engineering in Medicine and Biology Society (EMBS)	To establish technical standards for diagnostic, therapeutic, health-care, and bioinformation systems	https://www.embs.org/
U.S. Food and Drug Administration (FDA)—Center for Biologics Evaluation and Research (CBER)	To protect and enhance the public health through the regulation of biological and related products, including blood, vaccines, allergenics, tissues, and cellar and gene therapiesGuidance documents describe FDA's interpretation of our policy on a regulatory issue [21 CFR 10.115(b)]. These documents usually discuss more specific products or issues that relate to the design, production, labeling, promotion, manufacturing, and testing of regulated products. Guidance documents may also relate to the processing, content, and evaluation or approval of submissions as well as to inspection and enforcement policies. Guidance documents are not regulations and	https://www.fda.gov/about-fda/office-medical-products-and-tobacco/about-center-biologics-evaluation-and-research-cber https://www.fda.gov/vaccines-blood-biologics/guidance-compliance-regulatory-information-biologics

(*Continued*)

TABLE 82.3 (Continued)

Agency	Goal	Website
	alternative approaches may be chosen to comply with laws and regulations	
Medicines and Healthcare Products Regulatory Agency (MHRA)—The National Institute for Biological Standards and Control (NIBSC)	To play a major national and international role in assuring the quality of biological medicines through product testing, developing standards and reference materials and carrying out applied research. NIBSC supports health policy development and implementation through provision of expert evidence-based advice and technical support and are an important component of the Department of Health's risk management strategy for public health. NIBSC is designated as the UK's OMCL for Biological Medicines, working closely with MHRA and operating within the European regulatory network to carry out independent official batch release testing of certain types of product as required by EU law	https://www.nibsc.org/
European Medicines Agency (EMA)—Committee for Advanced Therapies (CAT)	CAT is the EMA's committee responsible for assessing quality, safety, and efficacy of ATMPs and following scientific developments in the field	https://www.ema.europa.eu/en/committees/committee-advanced-therapies-cat

As the tissue engineering field continues to rapidly expand in the US and across the globe, industry-wide standards development and coordinating bodies have formed to regulate tissue-engineered medical products and regenerative medicine therapies. *ARM*, Alliance for Regenerative Medicine; *ATMPs*, advanced therapy medicinal products; *NIST*, National Institute of Standards and Technology; *OMCL*, Official Medicines Control Laboratory.

cure a serious or life threatening disease. . .where preliminary evidence indicates the drug as the potential to address . . .such disease or condition." This need for a path to expedite therapeutic strategies that fulfill an unmet need (Fig. 82.1) is an important step forward for tissue engineering.

Future perspectives

The first generation of cardiovascular tissue engineering products has matured, and second-generation products are beginning to reach first-in-human use. Of 85 trials listed at the NIH clinical trial site (https://clinicaltrials.gov/) in August 2019, which were found using the search term "tissue engineering," only six utilize cardiovascular products as the intervention. However, these six include TEVGs and cardiac patches.

We expect future innovations in tissue-engineered cardiovascular products to be closely coupled to surgical improvements in cardiac care. Today, mechanical left-ventricular assist devices (LVADs) work efficiently to unload the heart and can serve as destination therapy either as a bridge to recovery or to transplantation [139]. Advances in LVAD technology combined with a better understanding of patient selection have led to unparalleled survival and a reduction in adverse events associated with these pumps. This in turn has led to the idea that efficient, safe LVAD systems can provide both the time and the clinical scenario needed for evaluating regenerative interventions. This "bridge to regeneration" would provide significant opportunities, for example, to improve the neovascularization of transplanted tissue-engineered constructs such as epicardial or septal patches. It could also provide a unique environment to evaluate electromechanical and vascular integration of larger full-thickness constructs or even to introduce whole hearts into the circulation. As biomanufacturing of quality-controlled decellularized whole heart scaffolds advances and industry improves procedures for generating and processing autologous pluripotent stem cells, we anticipate significant strides in these regards over the next decade.

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Chapter 83

Tissue organoid models and applications

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Introduction

One of the first documented cell culture experiments occurred in the early 20th century when Ross G. Harrison successfully cultured frog nerve tissue explants followed a few years later by Alexis Carrel and Montrose Burrows who proliferated cells from connective and epithelial tissue [1]. Since its introduction, cell culture has had a significant impact on research development regarding applications such as disease modeling, vaccinations, medicine, and human development and physiology. Recently, three-dimensional (3D) cell culture techniques have gained traction in various avenues of research due to its enhanced similarities to the microenvironment that cells experience in vivo. A subtype of 3D cell culture has been described as "organoids," although the actual definition of this term has been controversial since its introduction in the late 1900s [2]. For the purpose of this chapter, organoids will be defined as 3D culture models derived from primary or stem cells that are capable of self-organization and functionality similar to the tissue of origin.

Recently, there has been a resurgence of organoid models in literature according to PubMed citations including the term "organoid." As a matter of fact, since around 2010, there has been an exponential increase in publications involving organoids (Fig. 83.1). The recent increased interest in organoid models can be correlated with the enhanced culture methods and organoid models that can, to a degree, mimic functionality of human organs more precisely in vitro compared to standard two-dimensional (2D) culture and animal models. Similar to any 3D culture method, organoids provide an interactive 3D architecture for the cells, similar to an in vivo environment. Depending on the model, the extracellular matrix (ECM) that establishes the 3D architecture within the organoid can be self-secreted, exogenous, or a combination. Cellular behavior depends on these interactions that are generally lacking in 2D culture. 2D culture often shows altered cell behavior of monolayer culture on plastic [3,4]. Moreover, it is widely known that animal models, while generally beneficial in the clinical process, have proven to lack translational power for efficacy, safety, and toxicity in humans [5]. Organoids, on the other hand, have already shown to have excellent translational capabilities with drug analysis [6,7]. In the following sections, some of the current major organ models will be highlighted along with their cell sources and the current and prospective applications of these models.

Cell sources

Typically, organoids are derived from either primary adult cells or pluripotent and adult stem cells (aSCs). Improved isolation and culture techniques in the past decade have aided in propelling organoid research forward and developing models for various organs including heart [8], liver [9], lung [10], gut [11], retina [12], and pancreas [13]. Still, regardless of the improvements, these two major cell sources both have advantages and disadvantages regarding organoid culture.

First, primary adult cells are directly obtained from human tissue and are not immortalized. These cells are believed to have the greatest translational capability simply because they are unadulterated and are obtained directly from the source. Comparison studies to cell lines have shown primary cells to be superior in reflecting cell behavior regarding cell biomarkers, cytokine production and response, metabolism, and phenotypic characteristics [6,14–17]. For instance, a comparison between primary bronchial epithelial cells and common bronchial epithelial cell lines indicated major differences between differentiation potential, tight junction formation, and mucus production [18]. The utilization of primary cells in organoids

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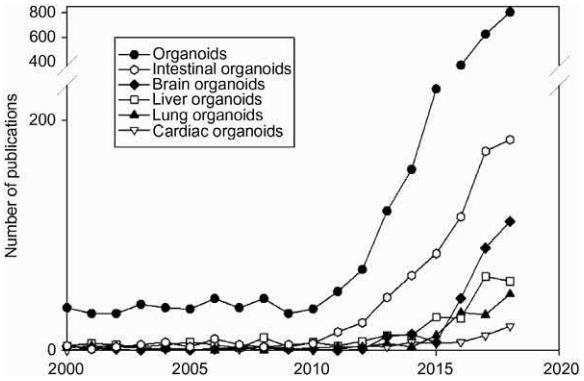

FIGURE 83.1 Number of articles published to PubMed including the organoid search term.

significantly enhances their translational capabilities regarding responses to drugs, infection, toxicity, and disease.

The major limitation of this cell source is its limited supply and expansion capabilities. Primary cells typically have slow growth and lose significant characteristics, viability, and proliferation after only a few passages. Various immortalization techniques have been investigated using transduction and oncogenes, but this genotypical alteration can lead to unwanted phenotypical change with differentiation and cellular function [6,19]. One methodology that has been investigated to solve this issue is the use of a Rho Kinase (ROCK) inhibitor, such as Y-27632, to prolong their expansion capacity [20,21]. For example, Gentzsch et al. demonstrated that bronchial epithelial cells cultured on a fibroblast feeder layer with Y-27632 were able to exponentially grow through 25 population doublings without significantly affecting functional characteristics [21]. While ROCK inhibitors have not shown to significantly alter phenotypical expression of cells, its impact has not fully been investigated for all cell types and may be causing undesirable changes. Another possible solution to the viability and prolonged functionality of primary cells has been organoids themselves. Deegan et al. demonstrated increased viability of hepatic cells in vitro in organoids when they typically cannot be maintained past 7 days on plastic [22]. Despite these improvements, the availability of primary cells significantly hinders their desired phenotypical properties.

The other major cell source for forming organoids has been the variety of stem cells available: aSCs, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs). Utilizing environmental and growth factors, the stem cells can be differentiated into the cells of interest for the specific organoid model. aSCs, or progenitor cells, are commonly used in a wide range of organoid models but are organ-restrictive. The advantage of aSCs is the rather simple protocol required for terminal differentiation into somatic cells. Unlike aSCs, ESCs are pluripotent stem cells (PSCs) that are isolated from the inner cell mass of blastocyst [23]. Their pluripotency allows them to differentiate into cells derived from all three major germ layers allowing for application in any organoid model. This property allows PSC-derived organoids to be excellent in vitro models for developmental biology and morphogenesis [24,25]. One of the major disadvantages that has been associated with ESCs is the ethical concern of their derivation from human embryos. As a result, iPSCs were developed through the reprogramming of adult somatic cells through transfection with ESC-associated genes: Oct3/4, Sox2, c-Myc, and Klf4 [26]. This induced pluripotency results in embryonic-like pluripotency providing comparable properties and applications to ESCs [27,28].

All of these stem cells have shown some capability to self-organize into organoid structures in vitro during their differentiation process and have been a defining feature of stem cell–derived organoids [24,29–31]. Unlike primary cells, the major advantage of stem cells is the potentially infinite expansion capabilities of the cells in vitro [32,33]. With the advancements in media and growth factors even aSCs have shown to have improved expansion. Furthermore, even though there is a limited supply of aSCs and ESCs, the advancements with iPSC development provide the opportunity for a limitless supply of cells for organoid fabrication [34]. Nevertheless, even though stem cell populations can differentiate into a myriad of cell types, the time and cost associated with differentiation is a setback. Another issue is ensuring complete differentiation and phenotypical expression matching the desired organ's cells, as this has not been fully quantified and confirmed for several differentiation protocols [35]. If the differentiation is not complete, the responses of these models could be affected. Overall, the availability of cells along with the desired application has typically dictated the cell type for each organoid model.

Types of organoid models

There have been a range of methodologies to produce varying structural organoids in vitro. For the purpose of this chapter, two main organoid models, or a combination of these models, will be reviewed: spheroidal models and ECM layered models. Spheroidal models, commonly known as "spheroids," are multicellular aggregates of cells that form a spheroidal shape due to some external stimuli [36]. While no exogenous ECM or 3D architecture is provided, this model allows for excellent cell–cell interactions and interactions with cell-secreted ECM. Moreover, spheroids have shown to exhibit chemical and cellular gradients similarly seen in vivo regarding waste, pH, carbon dioxide, oxygen, and drugs [37–39]. The first methodology developed to fabricate spheroids was the hanging drop method that utilizes the gravity within a

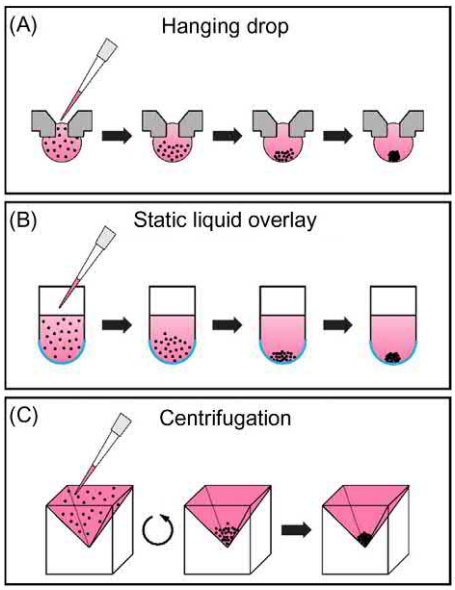

FIGURE 83.2 Spheroidal organoid fabrication methodologies. The three main methods to fabricate spheroids are by (A) the hanging drop method, (B) static liquid overlay with nonadherent surfaces, and (C) centrifugation in microwells.

hanging droplet of media (Fig. 83.2A) [40]. Beyond this method, other protocols have been developed to fabricate spheroids using centrifugation and the static liquid overlay technique (Fig. 83.2B and C) [36,41]. The multiple techniques allow for either mass or individualized production of spheroids. Extensions of this spheroidal model are the budding and branching models that can occur with stem cell and duct-like organoids. These appendages can be attributed to the self-organization of the cells representing the morphogenesis of the organ, forming structures, and ducts related to the organ of interest [42–44]. The second major organoid model is culturing cells or tissue fragments within or on a 3D architecture, typically a hydrogel, that is architecturally and compositionally similar to the in vivo environment [45,46]. The advantage of this added ECM component is the enhanced cell–environmental interaction as well as the cell–cell interactions in the previous models. In addition, it is typical to encapsulate spheroids within the hydrogel matrix to support organoid functionality and morphogenesis [8,47–49]. Thus it is common to see combinational organoid models that utilize any amalgamation of spheroids, ECM scaffolds, and tissue fragments. Finally, groups have utilized hydrogels and scaffolds to develop layered organoid systems to represent organs whose primary function involves interaction with the external environment such as the lung, gastrointestinal (GI) tract, and skin. To fabricate a representative model in these cases, Transwell culture systems have been widely utilized to allow for a multilayer system involving an apical epithelium and a basal stroma [10,50,51]. The major advantage of this layered system is the ability to remove the apical media for an air–liquid interface (ALI) that has shown to be crucial in epithelial cell differentiation and maturation [52–54]. Since most epithelial cells are continuously engaging with air in vivo, ALI culturing is the primary method to aid in recapitulating this physiological environment. In addition, this design allows for supplementary analyses that are otherwise unfeasible or insignificant in other designs. A common measurement of layered systems is the transepithelial electrical resistance to quantify the integrity and permeability of the epithelial or endothelial barrier. This measurement requires an apical and basal compartment to measure the resistance and voltage difference. Other experimental analyses for aerosolized and particulate scenarios are similarly not physiologically relevant in submerged systems and are severely altered in the medium regarding characteristics such as size, solubility, charge, and chemical properties [55].

Cardiac organoid

The heart is the first functional organ to develop in the human body and develops from the mesoderm [56]. When fabricating cardiac organoids, the main cell of interest has been the cardiomyocytes although some models have also utilized cardiac fibroblasts and endothelial cells. Cardiomyocytes are the muscle cells of the heart responsible for the contractile function that pumps the heart. Since primary cardiomyocytes are not readily available, the leading method of derivation is differentiation of PSCs. To obtain cardiomyocytes from PSCs, the main methodology has been to first reduce heterogeneity with bone morphogenetic protein (BMP) 4 and activin A followed by inhibition of Wnt signaling to induce cardiogenesis [57]. Precardiac organoids derived from PSCs demonstrated the importance of Bmp/Wnt signaling in the development of the two heart fields [58]. The main issue with differentiation is complete maturation that is required for drug cytotoxicity, toxicity, regeneration, and apoptosis sensitivity [59]. In addition, there are several subcategories of cardiomyocytes including ventricular cells, purkinje cells, and atrioventricular cells that could affect analysis [60].

Most of the cardiac organoids have been fabricated as spheroidal organoids [8,61–64]. The primary characterization of these cardiac organoids has been their capacity to "beat" similar to the organ. Devarasetty et al. fabricated spheroids from iPSC-derived cardiomyocytes using the static liquid overlay technique in nonadherent 96-well plates and were able to image and quantify this beating behavior [8]. In order to track specific organoids, they were encased within fibrin–gelatin hydrogel. The system was verified by quantifying the attenuation and stimulation

of cardiac beating through toxin and drug exposure. Another spheroidal model combined cardiomyocytes, cardiac fibroblasts, and endothelial cells for cardiac fibrosis modeling [64]. Besides the spheroidal model, variations of a microtissue organoid model have been developed by combining the cells with ECM [65–69]. A cardiac injury model demonstrated the innate regenerative capabilities of the cardiomyocytes after acute injury [68]. Another ECM-based model was able to deconstruct the pacemaker functions of the cardiomyocytes through imaging modalities [65]. Similar to the spheroidal model, the beating capacity of the cardiomyocytes can be observed but with the additional component of external stimulus in the ECM model. Each of these models has implications in the effects of external stimuli, such as drugs or toxins, on the viability and functionality of cardiomyocytes.

Liver organoid

The liver forms from the endoderm germ layer, and its primary functions of interest for organoid modeling are its synthesis and breakdown of molecules along with its metabolism of drugs [70]. Similar to cardiac organoids, the liver organoids have one primary cell of interest, the hepatocyte, with other secondary cells including Kupffer cells, hepatic stellate cells, and hepatic endothelial cells. Due to the improved viability of the usually fickle primary cells in organoids, it is common for both primary and PSCs to be utilized [71]. To differentiate PSCs into hepatocyte-like cells, one of the most efficient methodologies follows a strict timetable of activin A treatment, followed by BMP 4 and basic fibroblast growth factor (FGF) 2, then hepatocyte growth factor, and finally oncostatin M for mature hepatocytes [72].

Takebe et al. created liver bud organoids from iPSC-derived hepatic cells combined with human umbilical vein endothelial cells and human mesenchymal stem cells within a Matrigel hydrogel to model early organogenesis [73]. The self-organized buds showed gene expression similar to in vivo liver buds and were able to perform liver-specific functions and form functional vasculature upon transplantation into a mouse model. The analysis even showed metabolites specific to humans in the blood. Another spherical liver organoid system was capable of recapitulating glucose metabolism in normal and stressed conditions using hepatocytes [74]. The data collected illustrated more realistic sensitivity to the hormonal influences of insulin and glucagon that would be seen in vivo. For instance, high glucose stimulation resulted in lipid accumulation and formation of reactive oxygen species that has yet to be observed in 2D culture. Finally, one of the major interests of liver organoids has been their improved hepatotoxicity response to stimuli. A spheroidal model of primary hepatocytes, hepatic stellate cells, and Kupffer cells generated appropriate responses to common environmental toxins such as lead and mercury [62]. The utility of liver organoids in research is substantial as the organ processes most of the substances that enter the body.

Brain organoid

The brain is the major organ that develops from the ectoderm and has been modeled in a variety of organoids ranging from blood–brain barrier (BBB) models to cerebral cortex models. The BBB models have gained the most attraction in order to understand the regulation of this barrier and possible methods to bypass it for treating neurological diseases. Depending on the organoid model desired, there are six cells of interest in the brain: neurons, oligodendrocytes, microglia, astrocytes, pericytes, and brain endothelial cells [75]. It is typical of these organoids to either be primarily composed of PSCs or partly composed of PSCs. For instance, it is typical to differentiate neurons from PSCs using a variety of protocols for specific types of neurons such as cortical [76,77] or midbrain [78]. Similarly, differentiation protocols have been established for oligodendrocytes [79], microglia [80], astrocytes [81], and BBB endothelial cells [82]. Even certain brain regions can be generated through endogenous delivery of factors such as a functional adenohypophysis in 3D culture [83].

One of the first organoid models developed was capable of forming multiple regions of the brain from embryoid bodies (EBs) derived from neuroectoderm [84]. The model was especially significant as it did not use any growth factors to drive differentiation, but instead embedded the EBs into a Matrigel hydrogel that allowed for outgrowth. The final organoid contained markers for the following brain regions: forebrain, hindbrain, dorsal cortex, prefrontal cortex, hippocampus, and choroid plexus. This cerebral organoid provides an excellent model to study the neurodevelopmental processes involved between the neuroectoderm and cerebrum and possible mechanisms of neurological disease. On the other hand, one of the most complete organoid models developed has been a spheroidal model that contains all six main cell types of the brain (Fig. 83.3) [85]. This model utilizes the hanging drop method to form a spheroidal cerebral cortex encased in brain microvascular endothelial cells and pericytes to create a functional BBB. The advantage of this model is the interaction of all major cell types of the brain along with a highly functional BBB that is not reproducible in monoculture of brain endothelial cells. Recently, a similar BBB organoid model was established utilizing endothelial cells, pericytes, and astrocytes [86]. This model focuses specifically on using confocal fluorescence microscopy and mass spectrometry imaging to generate accurate,

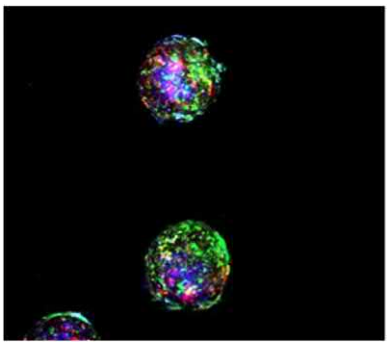

FIGURE 83.3 Multicellular cerebral cortex model with a center of neurons and astrocytes encased in an outer layer of microvascular endothelial cells and pericytes. *Reproduced from Chambers SM, et al. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol 2009;27(3):275, under CC BY licensing.*

scalable quantification of drug transport. Altogether these models provide a range of brain organoids that can be effective tools to elucidate developmental pathways and biological processes in the brain crucial for treatments.

Lung organoid

The lung and its associated airways originate from the endoderm and similar to the brain can be separated into subcategories: airway and alveolus models [87]. For the airway models the primary cell of interest is the bronchial epithelial cell that is capable of differentiated into Clara, goblet, and ciliated cells. Specialized media that is widely commercially available promotes this differentiation at ALI. The airway models are principally utilized for modeling related pathophysiologies with the cilia, mucus production, and hydration of the airways. For the alveolus models the primary cell of interest is the type I pneumocytes that cover more than 90% of the surface and facilitate the main function of gas exchange. Even though primary cells are typically used in most organoid models, PSC-derived airway epithelium can be obtained by inhibition of notch signaling [88]. Similarly, ESC-derived endoderm has been differentiated into a distal lung phenotype organoid using inhibition of the Hedgehog pathway [89]. There also exists the resident basal progenitor cells that are the stem cells of the airways and therefore have an application in airway organoids [90,91].

Rock et al. developed one of the first tracheospheroid models from isolated basal cells from the trachea [91]. This model was rather incomplete in differentiation as it lacked markers for mature Clara and goblet cells. Since then both tracheospheroid and bronchospheroid models have been optimized to discern the signaling cues for differentiation into the functional cells of the airway epithelium [92–94]. In addition, this has led to the establishment of the alveolospheres that are composed of type II pneumocytes [95]. This model demonstrated the stem cell capability of type II pneumocytes to self-regenerate and differentiate into type I pneumocytes. A more robust lung spheroidal model was developed through the differentiation of ESCs and iPSCs to create organoids with distinct epithelial and mesenchymal compartments [27]. The model had similar transcriptional profiles to human fetal lung suggesting translational capabilities to study lung development and disease. Another model generated lung bud organoids within a Matrigel hydrogel that developed characteristic branching airways and alveolar structures [96]. As mentioned previously, there has been considerable progress in developing layered organoid systems to provide an ALI structure similar to in vivo. Recently, a coculture organoid model of bronchial epithelial cells and lung fibroblasts was established to recapitulate airway remodeling [10]. The epithelial and mesenchymal cells were able to directly cooperate, and their combined interaction with exogenous transforming growth factor (TGF) β1 was analyzed histologically and transcriptionally. A tri-culture layered organoid model of bronchial epithelial cells, lung fibroblasts, and lung vascular endothelial cells was developed allowing for polarization of the epithelial layer and the three distinct layers of the airway: epithelial, mesenchymal, and endothelial [7]. These additional layered models provide the opportunity to examine the entry of aerosolized contaminants and toxins across the airway barrier compared to standard media spiking.

Gastrointestinal tract organoid

The GI tract develops in an anterior–posterior pattern from the endoderm into three sections: foregut, midgut, and hindgut [97]. From these three sections the entire GI tract is formed from the esophagus to the colon to the rectum. The three main specialized cells of the gut are the enteroendocrine cells that are responsible for secretion of GI hormones, the enterocytes that are primarily responsible for nutrient absorption, and the Paneth cells that secrete antimicrobial peptides. The cecum and the small intestine section of the GI tract have been of primary interest since they are the sites of the microbiome and nutrient absorption. The primary stem cells of the intestinal epithelium are the Lgr5 stem cells located at the bottom of the intestinal crypts [98]. Since the identification of these cells and their differentiation, they have been the primary stem cell source for intestinal organoids. For PSCs a combination of Wnt and FGF signaling has been known to differentiate the stem cells into midgut and hindgut fates [99].

Sato et al. fabricated one of the most complete intestinal organoids using Lgr5 stem cells in a Matrigel culture system [100]. The resulting self-organized organoid

contained all of the major cells of the gut, including enterocytes, enteroendocrine cells, Paneth cells, and goblet cells. When cultured for longer time periods, the organoid was capable of forming a mesenchymal layer with smooth muscle cells and fibroblasts. A significant issue that was noticed in this model though was the lack of a gradient for BMP signaling that should be apparent. Since then, Gjorevski et al. have optimized ECM matrices for the differentiation of stem cells into intestinal organoids in vitro without excessive exogenous factors [101]. Another gastric model was developed through temporal signaling of Wnt, BMP, FGF, retinoic acid, and epidermal growth factor [25]. The novel organoid model developed gastric glands and was capable of modeling *Helicobacter pylori* infection. A nutrient absorption small intestine organoid has also been fabricated using intestinal biopsies to create a layered organoid for apical and basal separation [102]. The microfluidic design showed epithelium with villi-like projections and enterocytes for nutrient absorption. Enzymes necessary for nutrient digestion were collected in the apical layer, further cementing the design as an excellent model for physiological digestion and absorption of nutrients. Another small intestinal organoid derived from mice expressed the presence of several important nutrient transporters and receptors for nutrient sensing, secretion, and absorption (Fig. 83.4) [103]. Other sections of the GI tract have similarly been modeled through organoid fabrication [104–106]. Each of these models is promising for mimicking not only normal physiological responses of the gut but also pathophysiological responses with the introduction of foreign bacteria.

Other organoid models

With the resurgence of organoids, there has been an expansion in the types of organs modeled with organoids beyond the major organoids listed previously. For example, pancreatic organoid cultures have been isolated from adult human tissue explants with endocrine differentiation potential [107]. The long-term expansion and endocrine functionality of the organoids have serious implications in beta cell replacement therapy. Takasato et al. derived kidney organoids from human iPSCs that were capable of nephrogenesis, forming distinct nephrons and ductal networks [31]. Recently, a 3D testicular organoid model was developed composed of spermatogonial stem cells, Sertoli cells, Leydig cells, and peritubular cells that was capable of hormone production with corresponding cell-specific gene expression [108]. The model was utilized to quantify the gonadotoxicity of common chemotherapy drugs and has since been used as a tool to characterize the effects of the Zika virus pathogenesis [109]. The testes organoid provides a more translational model for the human reproductive system that is not reflective in any animal model. Even less prominent organs, such as the retina, have an organoid model in development. A retinal organoid derived from ESCs demonstrated a species-specific difference in the optic cup between mouse and human [12]. Other organoid models include fallopian tubes [110], prostate [111], hair follicles [112], thyroid [113], tongue [114], skin [115], and thymus [116]. In the next decade, it is plausible that most organs will have developed some in vitro organoid model.

Applications

Tumor and disease models

A major limitation of cancer research has been the ability to accurately model tumor progression and phenotypes in vitro. Similar to previous statements, 2D culture fails to provide not only the 3D environment experienced in vivo but also the stiffness and surface topography that are crucial in cancer phenotypes [117]. While considered by many to be its own subcategory of organoids, tumor organoids can be fabricated from the isolated cells of cancerous tumors for various cancer types including prostate, colon, stomach, pancreas, and lung [105,118–121]. Pancreatic cancer organoids derived from human and murine models were shown to more adequately reflect the in vivo phenotype and cancer progression through proteomic and transcriptomic analyses [119]. Histological analysis of another pancreatic cancer organoid model displayed similar morphological features and differentiation markers to the tumor of origin [122]. Another major issue with current cancer research is the lack of the hypoxia experienced by tumors in vivo [123]. A glioblastoma organoid model was able to demonstrate this hypoxia condition

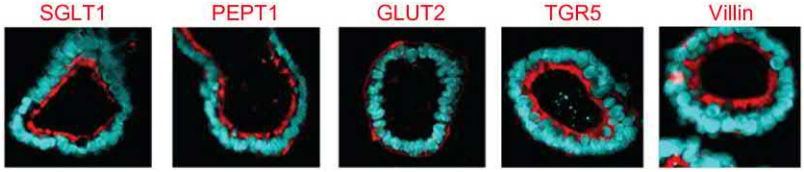

FIGURE 83.4 Small intestine organoid expressing key nutrient transporters (red), villin (red) expressed in microvilli, and the bile acid receptor TGR5 (red). Nuclei (blue) of cells were stained with DAPI. *Reproduced from Roberts DJ. Molecular mechanisms of development of the gastrointestinal tract. Dev Dyn 2000;219(2):109–20, under CC BY licensing.*

[124]. A hypoxic gradient was observed across the organoid with a characteristic hypoxic core. This same model demonstrated the cell heterogeneity that is observed with most cancers that are not recapitulated in vitro with other current methods. These improved tumor models could play a key role in the understanding of specific tumor progression and its changing behavior through chemotherapy and radiation therapy.

Beyond the development of tumor organoid models, other disease-specific organoid models have been investigated to aid in elucidating mechanisms specific to human disease. There are currently significant gaps in disease modeling regarding 2D culture and animal models due to the vast differences in animal biology and cell culture. Moreover, the simplicity of 2D culture and complexity of animal models create a difficult environment to collect quantifiable data about the diseases. Already, a variety of organoid models have been fabricated in order to recapitulate hereditary [84,125–133], acquired [93,96,134–136], and infectious disease [25,96,137–140]. Intestinal and lung organoids alone have been manufactured to model all three main types of disease. For instance, an intestinal model developed by Schwank et al. derived from cystic fibrosis cells was able to model the diseased phenotype using a forskolin-induced swelling protocol [141]. The organoid model was utilized to verify proof-of-concept gene correction with the novel CRISPR/Cas9 genome editing system. Likewise, nasospheroids have been fabricated with cells from a nasal biopsy of cystic fibrosis patients for a quantifiable robust model for cystic fibrosis regulator membrane protein activity [142]. Another lung organoid model derived from primary cells exhibited a fibrotic phenotype comparable to pulmonary fibrotic diseases after treatment with the stimulus TGF β1 [134]. A major breakthrough occurred with the most recent epidemical Zika virus using brain organoids. Cortical neurorganoids provided the first quantifiable evidence of the link between the virus and microcephaly with increased cell death, reduced proliferation, and reduced volume in virus-infected organoids [139,143]. Fabricating diseased organoids, whether hereditary, acquired, or infectious, could allow for improved molecular understanding resulting in improved treatment protocols.

Drug analysis

One of the most promising translational applications of organoids has been their capabilities for drug analysis. As mentioned earlier, 2D culture and animal models have proven to be inadequate in multiple occasions to recapitulate human physiology for drug testing. With drug development, 2D culture drug diffusion kinetics are not translational for human models, while animals' metabolisms vary significantly from humans [5,144]. Other unnatural characteristics of 2D culture include biochemical composition, culture-induced mutations, cell characterization, and surface tension [144,145]. These poor characteristics have played a role in the decrease in the development of drugs that has also been associated with a significant increase in developmental costs [146]. In multiple occasions, 3D culture alone has shown to be superior to standard 2D culture [147–149]. Organoid models, including tumor models, can provide a secondary or replacement model to determine if drugs have the efficacy and safety to reach clinical trials where most of the cost is incurred.

The primary organoid model for general drug analysis has been liver organoids due to the drug metabolic properties of the liver [136,150–152]. One study developed 3D liver microtissue organoids using primary hepatocytes and nonparenchymal liver cells for inflammation and drug toxicity testing in a 96-well plate format [150]. The liver model was able to recapitulate the hepatotoxicity that only occurs due to the interaction of inflammatory stimuli and the drug trovafloxacin. In comparison, 2D culture of hepatocytes did not illicit the same response. In addition, there have been disease- and cancer-specific studies, to quantify the direct interaction of drugs with the target organ [153–157]. In a cystic fibrosis rectal organoid model, physiological responses and restoration were observed with appropriate drug treatment [158]. Comparable studies have been completed with cystic fibrosis lung and intestinal organoids [159,160]. Some researchers have even investigated establishing organoids with patient-derived cells in order to develop personalized medicine (Fig. 83.5) [122,141,161–164]. In vitro expansion and analysis of patient-derived organoids can reveal patient-specific mutations and phenotypes allowing for more tailored treatments. As such, Mazzocchi et al.

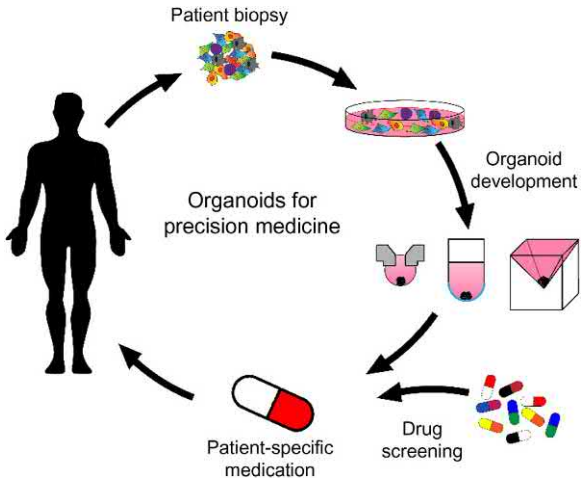

FIGURE 83.5 Personalized medicine concept utilizing patient biopsies to fabricate organoids to optimize patient-specific medication.

successfully microengineered mesothelioma tumor organoids from human patient tumor biospecimens [161]. This biofabricated model was able to demonstrate both specific tumor organoid drug responses along with biomarker testing for experimental drugs. Similarly, the same group demonstrated that patient-derived lung adenocarcinoma cells from pleural effusion aspirate behaved differently in tumor spheroidal organoids compared against 2D culture [120]. Chemotherapy drug resistance seen in the tumor organoid model was comparable to the donor's phenotype, while 2D culture of the same cells was susceptible to the same therapy. The 2D culture of the tumor-derived cells also lost their ability for tissue-like reorganization when grown on plastic indicating obvious phenotypical changes.

Organ-on-a-chip

One of the most common applications of organoid culture models in research has been its integration into a microfluidic device, known as an organ-on-a-chip (OOC), to simulate physiological fluid flow for exchange of nutrients and molecules. This supplementary component to the design strengthens the previous aforementioned applications of tumor and disease modeling and drug analysis. For example, tumor organoids integrated into microfluidic devices can simulate the metastasis and angiogenesis potential of tumors [165]. Other more specific organoid models, such as lung and GI tract, specifically benefit from the flow and mechanical capabilities of OOC platforms. The lungs are constantly interacting with the external environment with air flow that can contain pathogens, drugs, toxins, and other particulates. Varying levels of lung OOC designs have been fabricated for specific purposes that require the air interface and flow in the microfluidic design [166–168]. Huh et al. were able to establish a lung-on-a-chip that could simulate mucus plug ruptures and the associated cellular damage that can occur due to poor mucosal clearance like with cystic fibrosis [169]. Similarly, for the GI tract, Ramadan et al. developed a human GI tract OOC that allowed for flow of nutrients across its apical layer [170].

As OOC designs have cemented themselves in research, groups have integrated OOC designs together to create multiorganoid devices [7,171–174]. Multiorganoid integration further increases the significance of OOC designs, as tissues and organs are not isolated within the body; they interact with each other. Xiao et al. designed a five-organoid chip to recapitulate the female reproductive tract and its interaction with the liver [173]. The system successfully underwent a complete menstrual cycle with corresponding hormonal fluctuations and follicular maturation and differentiation seen in vivo. Moreover, there were significant phenotypical changes in the organoids in this multiorganoid system compared to static culture. These interactions between organoids are crucial in normal organ function, disease modeling, and toxicity analyses. For instance, multiple organoid designs provide a more complete system for tumor metastasis. A lung cancer metastasis multiorganoid chip was designed to investigate lung cancer metastasis to brain, bone, and liver [175]. The chip design successfully showed the transitional properties of the cancer cells and their invasive capacities to the organs. Finally, multiple drugs have been recalled or terminated due to secondary toxic effects on nontarget organs. Skardal et al. were able to demonstrate this characteristic in their three-tissue OOC platform of liver, heart, and lung (Fig. 83.6) [7]. Utilizing this model, they were able to quantify the secondary cardiotoxicity that is associated with bleomycin treatment through lung inflammation. This cardiotoxicity was not present in the isolated cardiac OOC design, thus requiring the interaction between the systems. The final aspiration of these microfluidic designs is to integrate enough OOC designs into one system, called body-on-a-chip, that can essentially mimic the entire physiological and metabolic functions and responses of the human body.

Developmental biology

An additional application of organoid models is specific to the stem cell–derived organoids and their potential in modeling human developmental biology and morphogenesis. Prior to these models, there was no human-derived model to adequately study human development except for

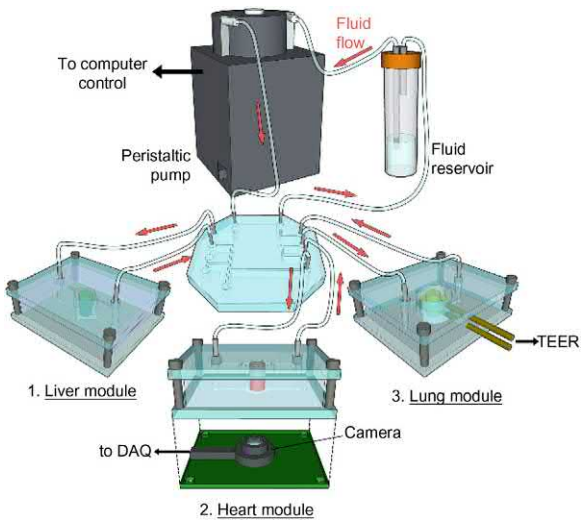

FIGURE 83.6 Multiorganoid microfluidic setup containing a liver organoid, heart organoid, and lung organoid connected in series with a peristaltic pump. *Reproduced from Hogan BL, et al. Repair and regeneration of the respiratory system: complexity, plasticity, and mechanisms of lung stem cell function. Cell Stem Cell 2014;15(2):123–38, under CC BY licensing.*

the limited access and limited progression of human embryos. There have been some developmental questions that have been impossible to answer until now due to ethical limitations [176]. With the development of organoid models, they have shown to recapitulate in vivo developmental stages in real time allowing analysis and quantification. This real-time progression of development has already been seen with varying levels of organoid models and has elucidated key signaling pathways.

McCracken et al. demonstrated the importance of Wnt/β-catenin signaling through the fabrication of gastric organoids derived from ESCs and iPSCs [177]. During the differentiation, continued activation of Wnt signaling was crucial in the formation of fundic organoids with stomach acid-producing cells capable of secreting digestive acids and enzymes. Likewise, sustained FGF signaling was critical for the nephrogenesis process in iPSC-derived kidney organoids [31]. Specific patterning of signaling played a role in the formation of segmented nephrons with distinct endothelial layers and renal interstitium. Even more intricate and specific organoids have been developed such as hair follicle organoids from progenitor cells [112]. While this model has been less investigated, it has shown the importance of plasticity of keratinocytes and inducibility of dermal cells in the formation of hair follicles. These are just a few examples of studies that have elucidated key developmental stages and signaling pathways. Other organoid models of lung [27,96], liver [178], brain [84], and pancreas [179] have shown similar potential. Through the differentiation of stem cells within the organoids, the actual signaling network that dictates development becomes apparent. Similar to drug analysis, organoid cultures can be developed with animal-derived cells to compare and contrast the developmental stages between humans and other animals. These studies are transforming the methodologies we employ to study human developmental biology and can have remarkable potential in this field and congenital diseases.

Conclusion

As detailed earlier, a variety of organoid culture methods are being investigated, and each shows promise in several translational areas. The types of organs modeled along with their complexity continue to grow. While primary cell sources provide the most characteristic cellular function, the continuing derivation and understanding of stem cells provide a more limitless supply of cells for scalable fabrication. Despite this rapid progress, there are limitations that have yet to be fully addressed with most models such as integration of immune cells and blood vessels and reproducibility of models. As immune cells circulate throughout the entire body, their incorporation is crucial in mimicking organ functionality to thoroughly model the organ's reaction to any type of stimuli, especially infection and inflammation. One of the most significant hurdles is mimicking the complexity and interaction within and between the organs as multiorgan models have illustrated the importance of their interaction. Still, the applications of these organoids are vast and highly impactful ranging from cancer modeling to infection and disease modeling to drug analysis. With the use of stem cells, further understanding of development biology, stem cell biology, and tissue regeneration can also be investigated. Patient-derived cells provide a more unique application, specifically for cancer and disease, with personalized medicine to determine the optimal treatment for each specific patient. The second major goal of organoid models appears to be a multiorganoid system known as the body-on-a-chip. While the complexity of this system is a major challenge, this platform could not only beneficially disrupt the drug development process, it can provide a more quantifiable method to study the interactions between major organs. As these models continue to advance, they are establishing themselves as easy-to-use products that have exceptional research and translational potential. Similar to general 3D cell culture, organoids are changing the current perception of cell culture and can dramatically change the development of science and medicine.

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Part Twenty three

Regulation, commercialization and ethics

Chapter 84

The regulatory process from concept to market

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Introduction

The US Food and Drug Administration (FDA) is responsible for regulatory oversight for a wide range of products, including food, medical products, products for veterinary use, and tobacco products. Within the FDA, the Center for Drug Evaluation and Research (CDER), the Center for Devices and Radiological Health (CDRH), and the Center for Biologics Evaluation and Research (CBER) are responsible for overseeing the regulation of human medical products which are drugs, devices, and biologics. The Office of Tissues and Advanced Therapies (OTAT) in CBER regulates a wide range of products, including gene therapies, cell-based products, tumor vaccines, human tissues for transplantation, xenotransplantation products, blood- and plasma-derived products, certain combination products, and certain medical devices. This chapter discusses issues relevant to cell-based products regulated by OTAT.

The 21st Century Cures Act, signed into law on December 13, 2016, outlines provisions to help accelerate medical product development [1]. Title III Section 3033 specifically applies to regenerative medicine therapies (RMTs) that can be designated as regenerative medicine advanced therapy (RMAT) if they meet certain criteria. RMAT designation offers a new expedited program to facilitate the development and review of certain RMTs intended to address an unmet need in patients with serious conditions [2]. Products that may be eligible for RMAT designation include cell therapies, tissue-engineered products, human cell and tissue-based products, and combination products using such therapies except those regulated under Section 361 of the Public Health Service (PHS) Act (42 U.S.C. 264) and Title 21 of the Code of Federal Regulations (CFR) Part 1271 (21 CFR Part 1271). Also included are certain gene therapy products, including genetically modified cells, which lead to a sustained effect on cells or tissues, and xenotransplantation products [2].

Section 3051 establishes an expedited review pathway for devices [3]. Section 3036 outlines provisions for FDA to collaborate with other government agencies and stakeholder to develop standards to support innovation for RMT products. These programs will be described in more detail next.

Cellular and gene therapy products are regulated as biologics. Therefore this chapter primarily discusses the investigational new drug application/biologics licensing application (IND/BLA) pathway. However, since biologics can be combined with or delivered by medical devices, some device-related issues will also be covered within these specific contexts.

Regulatory background

To understand the regulatory process, it is helpful to have some background knowledge of the relationship between laws, regulations, and guidance documents. Laws, which are enacted by Congress and signed by the President, provide the legal mandate for FDA's regulatory oversight. In many cases, FDA publishes regulations (rules) to

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implement a law's legislative mandates.[1] Rules can be modified by FDA over time to allow for changes in science or technology as long as the revised rule remains responsive to the legislative mandates. As an example, FDA published a revised rule regarding sterility testing for biological products [4]. This new rule provides manufacturers of biological products with greater flexibility and encourages the use of the most appropriate and state of the art test methods.

Unlike laws and regulations, which are both legally enforceable by FDA, guidance documents represent the Agency's current thinking on specific topics and are not legally enforceable. Guidance documents are intended to provide general advice to interested stakeholders and product developers (sponsors) on how to comply with certain laws and regulations. FDA issues guidance documents that provide advice on topics ranging from the regulatory process to the agency's scientific expectations for meeting regulatory requirements for specific product classes. For example, if FDA recognizes a need to provide guidance documents for developers of a specific product type, or for a specific clinical need, FDA may publish guidance documents that explain FDA's thinking on the applications of the regulations to those specific areas.

Over the last decade, FDA has published numerous guidance documents that are relevant to the development of cell-based products. These include guidance documents on cell manufacturing and characterization for cell-therapy products [5], on potency [6], as well as guidance documents that address FDA crosscutting issues, such as evidence of effectiveness [7]. On the public website, FDA updates information regarding newly published guidance documents and the guidance agenda for upcoming years [8]. Investigators may also wish to refer to other resources, such as OTAT Learn, a web-based educational series that provides outreach to OTAT stakeholders on a broad range of topics [9], or the CDRH Device Advice [10] webpage, which provides links to extensive regulatory information including CDRH Learn [11]. References to these and other resources, including draft[2] and final guidance documents, can be found at the end of this chapter.

Overview of development and approval process

The development of a cell-therapy product is an iterative process to establish the manufacturing process, characterize the product, and generate safety and efficacy data from clinical trials (Fig. 84.1) before the product can be approved and made available in the consumer market [12]. Before testing a cell-therapy product in humans the potential toxicity of a product must first be rigorously tested by well-designed in vitro and/or in vivo studies (i.e., preclinical testing). Once early-stage product development is successful, the sponsor submits detailed information regarding the manufacturing process and facility as well as data from preclinical testing and product characterization to seek an agreement from FDA to initiate a clinical investigation in humans (human testing). As illustrated in Fig. 84.1, human testing of investigational drugs is typically conducted in phases [from early, small-scale (Phase 1/2) to late-stage, large-scale studies (Phase 3)]. Each phase is considered a separate clinical trial and, after completion of a phase, the sponsor is required to submit their data to FDA for review, and the data are used to support a sponsor's proposal to continue to the next phase of studies. After the successful completion of clinical trials, a BLA can be submitted for permission to introduce or deliver for introduction, a biologic product into interstate commerce (21 CFR 601.2). After FDA has approved a product for marketing, FDA may require postmarketing studies as postmarketing requirements to gather additional information about the safety, efficacy (if the BLA was approved based on a surrogate or intermediate endpoint via the accelerated approval pathway), or use in a pediatric population. Up-to-date product approval information is provided on the FDA public webpage [13]. In the following subsections, detailed information regarding early-stage development, clinical trials, license application, and postmarketing surveillance are described.

Early-stage development

In order to successfully develop a cell-based product for marketing approval, the development program must address certain key issues: a well-defined product having consistent safety and quality attributes from lot to lot, which may be achieved through a reproducible, well-controlled, adequately scaled manufacturing process and establishment of product's critical quality attributes; preclinical testing incorporating proof-of-concept (POC) and toxicology studies that provide scientific rationale and support the proposed use of the product; clinical evidence of effectiveness; and clinical evidence of an acceptable safety profile weighed against the benefit of the therapy.

1. The Government Printing Office annually publishes all current regulations (rules) in the CFR (in print and electronically). The CFR is divided into 50 titles or subject areas, and Title 21 pertains to food and drugs. Specific parts of CFR are referred to throughout this chapter and the Appendix to this chapter provides a summary of the parts of Title 21 that are most closely related to material in this chapter.

2. Draft guidances are published for comment and are not for implementation until finalized. Stakeholders should contact the relevant product office or division for the most up-to-date agency perspective on an issue discussed in draft guidance.

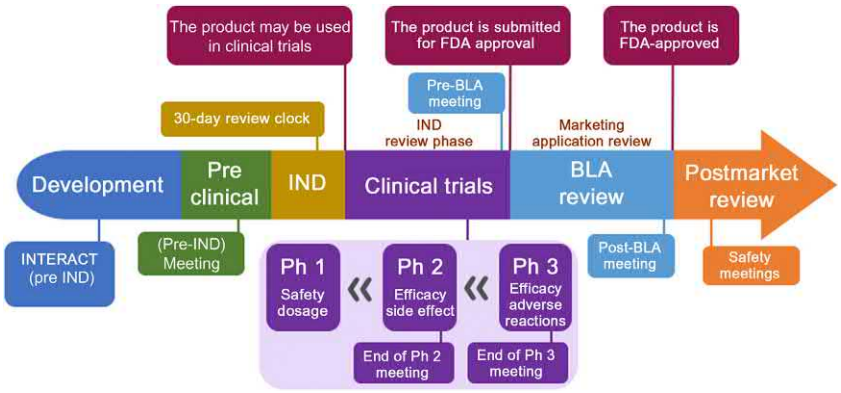

FIGURE 84.1 Interactions with FDA throughout the product lifecycle. Product development is an iterative process, with frequent FDA and sponsor interactions. Ph, Phase; "«" denotes the increase of clinical trial sizes from Phase 1 to Phase 2 to Phase 3.

Consideration of each of these key areas at an early stage may improve the overall efficiency of product development.

Chemistry, manufacturing, and controls

There are several key CMC (chemistry, manufacturing, and controls) issues to consider early in the development of a cell-based product, including sourcing of the biological material used for product manufacture, cell banking strategy, identification of critical cellular product quality attributes, development of a sufficiently robust manufacturing process to support clinical testing under an IND, and whether or not the cellular material is a component of a combination product.

When the cell-based product to be delivered consists of cells not derived from the recipient, it is necessary to perform a donor eligibility determination that involves both screening and testing of the selected donor to adequately and appropriately reduce the risk of transmitting relevant communicable disease agents and diseases (21 CFR Part 1271 Subpart C) [14–21]. In accordance with 21 CFR 1271.155, sponsors may submit to FDA a request for an exemption from or alternative to any of the donor eligibility determination requirements found in 21 CFR 1271 Subpart C [22]. Generally, a separate exemption request must be made at both the IND phase and when filing a BLA. It is important to consider the issue of donor-sourced biological material and donor eligibility requirements early in the development program including the use of human cell banks and cell lines since incomplete or insufficient information could necessitate additional product safety testing.

The strategy for manufacturing a cellular product that is initiated using starting material from a characterized source of cells may, in some cases, involves the establishment of a two-tiered cell bank, the master cell bank (MCB), and the working cell bank, which is derived from the MCB [23]. Early consideration of a cell banking program capable of supporting aspects of both preclinical and clinical development is important. In the event that it is necessary to establish new cell banks during product development, plans should be in place to demonstrate their comparability to the cell banks used previously for product manufacture.

Comprehensive cell characterization is a cornerstone in the development of cell-based products. A multiparametric analytical testing approach designed to assess distinguishing critical quality attributes of a cell-based product will help meet regulatory expectations for demonstration of product identity, purity, and potency. Characterization of the cellular product serves to inform the development of an overall product testing strategy and establishment of appropriate acceptance criteria to ensure product quality. Careful efforts devoted to cellular product characterization during the initial stages of premarket development may aid in the detection of subpopulations of cells in the final product that could present safety concerns requiring the implementation of mitigation strategies such as target cell selection/enrichment or off-target cell depletion to manage any risks posed to recipients.

Early attention to details associated with the design of a robust manufacturing process is recommended. Consideration should be given to the feasibility of reproducibly generating cells that possess the desired critical quality attributes at a scale capable of producing sufficient cell numbers to support overall product development. This includes assessing the quality and accessibility of reagents crucial for the execution of the manufacturing process. It should be noted that unanticipated changes in critical reagents could have a negative impact on product quality.

Pharmacology and toxicology

Prior to initiation of a clinical trial, a sponsor will need to provide adequate data generated from pharmacological and toxicological studies to establish that it is reasonably safe to conduct the proposed clinical investigation. The results

of these studies provide data critical to (1) establish the scientific rationale and biological plausibility of the proposed approach (i.e., demonstration of POC); (2) identify and characterize potential local and systemic toxicities, including the time frame for onset (i.e., acute vs long term), incidence, severity, and transient or chronic nature of the findings; (3) determine a possible dose−response relationship for guiding dosing for the planned clinical trial; (4) support subject eligibility criteria; and (5) identify physiologic parameters to help guide appropriate clinical monitoring [24].

Multiple in vitro and in vivo studies, spanning several iterative prototypes of the product and delivery device, may be necessary to translate a cell-based product from the bench to the initial clinical trial. During this process the incorporation of key features into the design of early discovery-phase animal studies may maximize the relevance and interpretability of collected data and facilitate translation to the clinic. For example, if safety endpoints are incorporated into discovery-phase studies, it may be possible to identify potential safety concerns early in product development, which can then be managed (such as through changes to product design or manufacturing) and investigated further in subsequent preclinical studies. Similarly, archiving relevant biospecimens during preclinical development allows for the opportunity for subsequent analysis if unanticipated adverse events (AEs) arise in later studies, which may help identify the cause of an observed toxicity. This, in turn, may inform the design of appropriate follow-on investigations.

For cell-based products, early investigation into cellular distribution, cell fate, host response, and the rate of resorption/degradation for products that contain a scaffold or matrix may be beneficial. As the target tissue undergoes remodeling, the structural properties, donor and host cell activity (such as migratory activity, proliferation, and phenotypic changes), and the rate of tissue in-growth will likely change. An understanding of these in vivo parameters and their impact early on in product development will help identify appropriate endpoints, outcomes measurements, and study durations for definitive preclinical studies. For example, if the product is designed to provide mechanical support or structure, an assessment of biomechanical properties and function at multiple time points during degradation or remodeling may be particularly important. In some instances, it may not be possible to conduct complete functionality testing of a product prior to implantation, as it may undergo remodeling in vivo with accompanying changes in biomechanical performance. Appropriate characterization of this parameter following implantation would then be an important consideration when designing the POC and safety studies.

Some additional early considerations for preclinical development may include selecting a biologically relevant animal model, maintaining cell survival in vivo, the use of appropriate control groups, and assessment of in vivo safety and activity.

Clinical

Development of novel cell-based products is an iterative process. Therefore it may be prudent for the sponsor to develop a target product profile. This is a "living" document that should be updated periodically as even well-considered investigational plans are likely to need revision as additional data are acquired. Nonetheless, early thought to the tentative design of the clinical studies can provide a framework for the overall development program, including preclinical studies. Preclinical studies usually mimic the clinical situation as closely as possible; therefore coordinating the preclinical and clinical programs may help improve efficiency. For example, if a preclinical animal study uses a specific delivery procedure, and then it is decided that the first-in-human will use a different delivery procedure, additional preclinical studies may be necessary to support the safety of the new clinical procedure. Early phase studies generally have a primary objective of assessing safety. However, primary or secondary objectives usually include bioactivity assessments. Such preliminary activity data can help guide the subsequent development program.

When selecting the study population for the first-in-human study trial, sponsors should consider the target indication, interpretability issues, and the risk of the study procedure. A product that can potentially cause long-term adverse effects is usually felt to have an unacceptable risk when administered to normal healthy volunteers. Therefore first-in-human studies for cell-based products usually enroll a study population of subjects who have a specific disorder. When possible, such studies should be conducted in individuals who can understand and consent to the study procedures and risks (see Section 12B Clinical Research Involving Children).

Selection of the number of subjects that will be enrolled in an early phase study should take into account the characteristics of the study population to include age and comorbid conditions, the cell-based product, and potential long-term effects. Enrolling a relatively small number of subjects avoids exposing a large number of subjects to risk, while allowing for the collection of preliminary evidence of safety.

In the absence of preliminary evidence of safety for a single dose and data regarding the time course for bioactivity of the biologic, repeat dosing may represent an unreasonable risk. Therefore a single-dose regimen is appropriate for most first-in-human trials. In addition, prior to the collection of preliminary safety data for the product, a first-in-human study should avoid concurrent administration of the

product to multiple subjects. Staggered administration to sequential subjects provides an intersubject interval to monitor for acute and subacute AEs. This approach may prevent multiple subjects from experiencing an AE related to the product.

The safety monitoring plan should include assessments that can capture early, intermediate, and delayed AEs that may be expected, based on preclinical and clinical data, as well as on theoretical concerns.

Stopping rules are criteria, usually based on a number or frequency of specific AEs, that if triggered would temporarily halt the study, pending a safety review. Stopping rules should be considered for all studies and may be required for higher risk studies.

US Food and Drug Administration/ sponsor meetings

OTAT holds informal and formal meetings with sponsors prior to and during product development as illustrated in Fig. 84.1. There are various formats for meetings with FDA, including written responses, teleconference, or face-to-face. There are several different types of formal meetings that can occur between OTAT and sponsors. These meetings often represent critical milestones in the product development program, and each is subject to different procedures.

For sponsors developing novel investigational products, using innovative or complex manufacturing technologies, or using cutting-edge testing methods who are at an early stage of product development, CBER offers an informal meeting, Initial Targeted Engagement for Regulatory Advice on CBER products, also known as the INTERACT program [25]. In addition to these early interactions, additional presubmission and submission-related meetings provide sponsors the opportunity to obtain nonbinding feedback from FDA on specific questions prior to submission of an IND or BLA. Such meetings may help sponsors to avoid missteps or delays that may be costly in terms of time and financial resources.

A meeting package that includes targeted, thoughtful, and clearly articulated questions, as well as a detailed summary of information, facilitates a productive meeting. Information about what should be included in meeting packages, meeting timelines, and meeting procedures can be found in FDA guidance documents [26–28], the OTAT Learn webinar series, or by contacting the Division of Regulatory Project Management in OTAT. The Office of Communication, Outreach and Development (OCOD)[3] at CBER also serves as a liaison with CBER components to provide advice and assistance to manufacturers and scientific associations to promote their understanding and compliance with FDA regulations.

Submitting an investigational new drug application

An IND is required (21 CFR 312) prior to clinical testing of a cell-based product that has not been approved by the FDA. A complete IND package consists of all required forms and sections, as specified in 21 CFR 312.23(a) and itemized in the checklist on page 2 of Form FDA 1571. A more detailed description of the key information that should be submitted with the original IND application is provided in the following subsections.

Required US Food and Drug Administration forms

Forms 1571, 1572, and 3674 should be included in an IND submission. These forms can be found on the FDA website, along with more detailed instructions for completing the forms [29,30].

Form FDA 1571 is a formal contract indicating that a sponsor will adhere to IND regulations to include requirements for informed consent, obtain institutional review boards (IRB) review of the IND, and comply with the IND regulations. FDA 1571 also contains basic administrative information regarding the sponsor, investigational drug, and proposed clinical trial [e.g., indications(s), phase of investigation, data monitoring] and includes a checklist of submission components. A signed Form FDA 1571 is required for all original IND submissions. For a sponsor–investigator IND, the sponsor–investigator should be named and must sign the form. For an IND sponsored by a pharmaceutical firm or research organization, the name of the sponsor's authorizing representative should be entered, and that individual must sign the form.

Form FDA 1572, the Statement of Investigator, is an agreement that an investigator will provide certain information to the sponsor and will comply with FDA regulations. This form serves the dual purpose of enabling the sponsor to establish and document the qualification of the investigator and clinical site, and informing the investigator of his/her obligations. Although it is the IND sponsor's responsibility to obtain a signed Form FDA 1572 from each investigator [21CFR 312.53(c)], the FDA does not require that Form FDA 1572 be submitted to the Agency. However, since Form FDA 1572 collects all the information that must be submitted to the FDA as per 312.23(a)(6)(iii)(b), many sponsors choose to submit

3. Consumer Affairs Branch (CBER): ocod@fda.hhs.gov.

Form 1572 along with the curriculum vitae of the investigator(s) [31].

Form FDA 3674, "Certification of Compliance with Requirements of ClinicalTrials.gov Data Bank," ensures compliance with the requirement for registering and reporting results from human clinical trials of human drugs (including biological products) and devices to the clinical trials databank [32].

Investigational new drug application contents

In addition to the forms described above, all IND submissions must include a table of contents, an introductory statement, and a general investigational plan. An Investigator's Brochure (IB) is required when clinical investigators at multiple clinical sites are participating in a protocol conducted under the IND. An IB is not required for single-site, sponsor−investigator-initiated INDs [21 CFR 312.55(a)]. Information to be contained in an IB is described in 21 CFR 312.23(a)(5). A well-organized IND submission includes distinct, stand-alone sections for the clinical protocol, product/CMC information, preclinical pharmacology/toxicology information, and other sections as described below. The electronic common technical document (eCTD) represents the standard format for submitting applications to FDA/CBER [33,34].

Previous human experience/electronic common technical document Module 2.5: clinical overview

The IND should include detailed summaries of any available safety and efficacy data from previous clinical experience with the study agent or related products. The FDA will consider this safety and efficacy data, along with the comparability of the study products, the study populations, and the delivery procedures, when reviewing the IND.

Chemistry, manufacturing, and controls information/electronic common technical document Module 3: quality

The Quality section of the IND contains information about the manufacture of the investigational product, including details about the starting material and reagents used in the manufacturing process to produce the drug substance (DS) and the drug product (DP) [5,35]. The DS is composed of the active pharmaceutical ingredient (e.g., a target cell population identified by specific cell surface markers or a specific cell phenotype) and is intended for further manufacturing to produce DP. The DP represents the final formulated material intended for administration. Final formulated DP is derived from a single or multiple DS and any added excipients used to constitute the final formulated investigational cell-based product. For manufacturing process steps that use materials and reagents of human or animal origin, it is necessary to provide documentation of compliance with 9 CFR 113.53: Requirements for ingredients of animal origin used for the production of biologics. The CMC information should include a description of the safety and quality attribute testing performed on the final product prior to release for the administration to study subjects. The CMC information typically provided in IND includes, but is not limited to

- description of starting material(s), reagents, and excipients, including safety and quality of these materials; if starting material is from an allogeneic donor, information about the eligibility of the donor (i.e., donor screening and testing);
- details of the manufacturing process and manufacturing controls;
- details of in-process testing performed at key stages of the manufacturing process;
- characterization of DS and DP for assessing their safety and quality including specifications which include specific test methods and acceptance criteria;
- data pertaining to the stability of formulated DP in the final container;
- information regarding the intermediate and final product storage containers and optimal storage conditions;
- container labels; and
- procedures for tracking the investigational product at each step throughout the manufacturing process.

Pharmacology and toxicology data/electronic common technical document Module 4: nonclinical

FDA determines whether the pharmacology/toxicology data submitted with the IND provide sufficient evidence that the investigational product is reasonably safe for initial testing in humans [21 CFR 312.23 (a)(8)]. For each preclinical study intended to support the safety and rationale of a proposed clinical trial, a complete study report should be provided that includes (1) a prospectively designed protocol, (2) a detailed description of the study performed (i.e., description of animal model, control and test articles used, dose levels, detailed procedures for test article administration and collection of all study parameters), (3) results for all parameters evaluated (i.e., individual animal data as well as summarized and tabulated results), (4) an analysis and interpretation of the resulting data, and (5) a statement of compliance with good laboratory practice regulations (21 CFR Part 58).

Clinical protocol/electronic common technical document Module 5: clinical

An IND submission should contain a clinical protocol for each planned study. The complexity of the protocols will vary with the phase and size of the planned clinical investigation; generally, some key elements of the study design include the study objectives, proposed study population, dose and regimen, treatment plan, monitoring plan and follow-up, and planned assessments/endpoints and analyses.

Additional information

All other pertinent information may be incorporated under various regional information and literature reference headings within the relevant eCTD module (i.e., Administrative Information, Quality, Nonclinical Study Reports or Clinical Study Reports, delivery device information and data). Additional supporting information could consist of copies of relevant publications, plans for assessing safety and effectiveness in a pediatric population, certificates of analysis obtained from manufacturers/suppliers of nonclinical grade reagents, and copies of meeting minutes from pre-IND interactions. In circumstances when information pertaining to the investigational product has been wholly or partially submitted to the FDA in previous premarket submission(s) and/or master file (MF)(s), a letter of cross-reference authorization may be provided in Module 1 Administrative Information of the IND submission. A letter of cross-reference authorization grants FDA permission to review specific proprietary details contained in the cross-referenced files to support other premarket or marketing applications. The letter of cross-reference authorization is provided by the sponsor/holder of the cross-referenced IND (s) and/or MF(s) and should refer to specific information and sections of the IND and/or MF that are authorized to access for review.

US Food and Drug Administration review of an original investigational new drug application submission

When a sponsor submits an original IND, the FDA reviews the submission within the next 30 calendar days. Unless the FDA imposes a clinical hold, the clinical studies described in the original IND submission may be initiated at the conclusion of the 30-day review period. A clinical hold is an order by the FDA to delay a proposed clinical investigation or to suspend an ongoing study. When FDA review of an IND results in a clinical hold, the sponsor receives a clinical hold letter within 30 days of being notified that the IND is on hold. The clinical hold letter summarizes the deficiencies that caused the clinical hold to be enacted.

The IND regulations outline the general reasons for which an IND can be placed on clinical hold (21 CFR 312.42). A retrospective analysis performed by FDA staff evaluated clinical hold letters issued over a 3-year period. This analysis identified some common reasons for clinical hold of INDs regulated by OTAT including CMC deficiencies (donor eligibility determination, MCB qualification), insufficient information to assess the risks to study subjects, and issues with the proposed study population or procedures (e.g., unacceptable risk associated with performing the investigational procedures in the proposed study population) [36].

Later-stage development topics

Compliance with current good manufacturing practice

Similar to other aspects of product development, the current good manufacturing practice (cGMP) requirements increase over the course of product development. For Phase 1 studies, investigational cell−based products are exempt from the cGMP regulations in 21 CFR Parts 210 and 211, with certain exceptions [37]. However, to ensure product safety, FDA has issued a guidance document regarding cGMP requirements that addresses sterility assurance, quality oversight, and facilities control during Phase 1 studies [37]. cGMP regulations in 21 CFR 211 apply to cell-based products in Phases 2 and 3 clinical studies. Adequate documentation and records management should also be in place for all phases of clinical study.

Because cGMP applies to both the manufacturing process and the facilities, it is important to note that many elements of product manufacturing and characterization and cGMP compliance cannot be separated and should develop concurrently. Examples of cGMP activities that may develop over time as clinical development progresses include process improvements and process validation, as well as analytical methods development and validation. Process controls and acceptance criteria should be further refined prior to Phase 3 based on knowledge of the manufacturing process and critical product attributes related to safety and efficacy. Sponsors can request a meeting with CBER's Office of Compliance and Biologics Quality (OCBQ), specifically with the Division of Manufacturing and Product Quality (DMPQ), to obtain specific advice on facility design, process controls, equipment qualification, and process validation.

Product readiness for Phase 3

To initiate Phase 3 clinical studies that are intended to provide substantial evidence of effectiveness and safety, it

is necessary to have in place a consistent, reproducible, and adequately controlled manufacturing process. Standard operating procedures that direct each stage of the manufacturing process should be finalized prior to Phase 3. Before initiating a Phase 3, pivotal clinical trial, analytical assays for process intermediate(s), and final product evaluation should have been identified, developed, qualified, and implemented. Acceptance criteria for these assays should be established and the range of accepted variation appropriately set to reflect testing results accumulated through prior manufacturing experience. Any changes introduced at this point in product development should be conservative with respect to any overall impact on the manufacturing process and product quality. Representative examples would be the modification of procedures to increase production capacity or instituting a change in the supplier of a key reagent. Final product formulation, storage conditions, as well as shipping and handling procedures should be established prior to initiating Phase 3 clinical testing.

Potency assay

By the time Phase 3 clinical testing is initiated, an assay or matrix of assays developed to measure product potency should be identified, qualified and incorporated into the panel of final product release tests [21 CFR 600.3(s)]. The results from potency testing provide important information that relates directly to manufacturing process consistency and reproducibility, attest to the comparability of products manufactured using starting materials obtained from different donors, is valuable for assessing the impact of changes made to the manufacturing process on final product critical quality attributes, and provides evidence of product stability under established storage and shipping conditions.

The most appropriate assessment of cell-based product potency related to its propensity to elicit a relevant clinical effect will be determined by identifying quality attributes specific to the product and the clinical indication for which it is intended. Quality attributes related to potency may include intrinsic molecular, biochemical, immunologic, phenotypic, physical, and biological properties of the cell-based product. Accordingly, the FDA assesses the suitability of a specific potency assay on a case-by-case basis in consideration of the factors mentioned.

Cell-based products are characterized by dynamic biological properties that present significant challenges to the development of a reliable, quantitative potency assay. As such, development of a potency test for these products is done frequently in a step-wise, incremental approach that allows for its modification as new information about the product characteristics, including mechanism of action, is discovered during the process of clinical development. In conjunction with other lot release tests the potency assay is intended to demonstrate that only those manufactured product lots which meet predefined acceptance criteria are administered to enrolled study subjects throughout the entire life-cycle of product development [6].

It may be the case that a single biological or analytical assay does not provide an adequate measure of product potency, and it could be necessary to develop a matrix of complementary assays that assess different product attributes, each affiliated with product quality, consistency, and stability to demonstrate product potency. The potency assay matrix could include testing for relevant nonbiological attributes such as mechanical and biophysical properties that are important for product function, and the matrix approach does not require all assays to be quantitative.

Pharmacology and toxicology

Preclinical studies to support the safety of the first-in-human clinical study are typically completed prior to submission of the IND. However, additional animal studies may be necessary in certain circumstances later in development. For example, depending on the product type, target population, and any concerns that arose during earlier phases of testing, reproductive and developmental toxicity studies may be needed to support licensure. These studies can usually be conducted concurrently with Phase 3 trials [38]. In other instances, additional nonclinical studies may be necessary prior to initiation of later-phase trials, such as following any significant changes to the dose level(s), dosing regimen, or target population. Similarly, if there is a modification to the manufacturing of the product, the comparability of the later-phase product to the product evaluated in earlier-phase clinical trials may be unclear. In this case, bridging nonclinical studies may be necessary to establish comparability. After clinical development has begun, additional nonclinical studies are sometimes warranted to provide insight into the in vivo pharmacology and mechanism of action of the product. For example, preclinical data may aid in the identification of an appropriate potency assay for Phase 3.

Phase 3 clinical development

By the time the Phase 3 clinical studies are planned, it is important for sponsors to understand some of the key issues that inform the design of Phase 3 trials. Early-phase trials offer an opportunity to optimize study procedures in terms of product delivery, dose regimen, and concomitant treatments and to identify potential safety concerns as well as gather early evidence of activity. Early-phase clinical studies can also provide an

understanding of the appropriate study population, the treatment effect size for the Phase 3 primary endpoint, the time frame for product bioactivity, and the durability of response.

When submitting a Phase 3 protocol, the FDA recommends that sponsors consider submitting a request for a Special Protocol Assessment (SPA) [39]. Concurrence on a protocol under the SPA program is an agreement between the FDA and a sponsor on the design and size of a Phase 3 trial that is intended to provide evidence of effectiveness.

Combination products

A combination product is composed of two or more different types of medical products (e.g., drug−device, device−biologic, drug−biologic, and drug−device−biologic). Under Title 21 of the CFR Part 3.2(e) [21 CFR 3.2(e)], combination product includes

1. a product comprised of two or more regulated components, that is, drug/device, biologic/device, drug/biologic, or drug/device/biologic, which are physically, chemically, or otherwise combined or mixed and produced as a single entity;
2. two or more separate products packaged together in a single package or as a unit and comprised of drug and device products, device and biological products, or biological and DPs;
3. a drug, device, or biological product packaged separately that according to its investigational plan or proposed labeling is intended for use only with an approved individually specified drug, device, or biological product where both are required to achieve the intended use, indication, or effect and whereupon approval of the proposed product the labeling of the approved product would need to be changed, for example, to reflect a change in intended use, dosage form, strength, route of administration, or significant change in dose; or
4. any investigational drug, device, or biological product packaged separately that according to its proposed labeling is for use only with another individually specified investigational drug, device, or biological product where both are required to achieve the intended use, indication, or effect.

In certain instances, a cell-based product may be regulated as a combination product. This includes cellular (biological) components that are physically or chemically combined with a drug and/or device. Cellular components physically or chemically combined with devices are often in the configuration of a "cell-scaffold" construct. In such cases, sponsors will need to develop a testing strategy that assesses the final combination product construct as well as key safety and quality attributes of the individual components brought together to create the combination product [40]. Cell-based products may require administration to a patient by a specific type of delivery device, which may constitute a cross-labeled combination product. In these cases, it is important to demonstrate the compatibility of the cell-based product with the delivery device, that is, delivery of the product using the device does not adversely affect cell-based product quality (e.g., viability, function, delivered dose, etc.). For any combination product, each constituent part retains its regulatory status (as a biologic or device, for example). As a result, the differences in regulatory pathway and requirements for each component can impact aspects of the lifecycle of the combination product, including its preclinical testing, clinical investigation, marketing application, manufacturing and quality control, AE reporting, labeling and advertising, and postapproval modifications.

In general, combination products are assigned to the FDA center that will have primary jurisdiction for its premarket review and regulation. Consistent with Section 503(g) [1] of the FD&C, assignment to a center with primary jurisdiction for premarket review and postmarket regulation is generally based on a determination of the "primary mode of action" (PMOA) of the combination product. For example, if the PMOA of device−biologic combination product is attributable to the biologic component, such as cellular and gene products, CBER would have primary jurisdiction over the combination product because CBER is responsible for premarket review of that biological product. In the case of a catheter device that is designed to deliver cellular therapies as another example, although the biologic and the device need to be studied together to mediate the intended therapeutic effect, it would likely be determined that the cells provide the PMOA; therefore CBER would lead the review of the combination product and CDRH would provide consultative review for the delivery catheter.

For combination products that include biological products and human cells, tissues, and cellular and tissue-based products (HCT/Ps), a manufacturer of a combination product must comply with the cGMP requirements that would apply to the biological products or HCT/P if it were not part of a combination product. For HCT/Ps, current good tissue practice requirements outlined in 21 CFR 1271 would apply in a complementary manner. The manufacturer should also comply with drug cGMP and device quality system regulation requirements as applicable in accordance with 21 CFR part 4 [41]. CBER and CDRH often collaborate in the premarket review of combination products that contain both device and biological components. Regarding the device component (e.g., a scaffold or delivery device), insight into considerations for product manufacture, biocompatibility, electrical safety, electromagnetic

compatibility, software validation, and physicochemical and mechanical testing may be obtained by review of FDA Guidance documents and the published summaries of cleared 510(k) and approved premarket applications (PMAs). FDA has published guidance documents that provide current thinking on approaches for evaluating the biological responses to biomaterials and material sourcing issues [42], as well as physiochemical analyses and preclinical tests that may be appropriate for scaffolding materials [43]. A review of relevant CDRH-recognized consensus standards may also be helpful [44].

Tissue-engineered and regenerative medicine products

Many of the tissue-engineered and regenerative medicine (TE/RM) products in the market or currently under development are products in which cells are combined with a biomaterial scaffold [45]. The scaffolds usually serve as the matrix that provides mechanical and chemical support for tissue regeneration. One of the regulatory challenges for a cell-scaffold product lies in the fact that the final product combines distinct components that are prepared through disparate manufacturing processes. This complexity necessitates a thorough characterization of individual components as well as the characterization of the assembled final product to ensure the safety and effectiveness of the final TE/RM product. If degradable biomaterials are used as structural scaffolds or for encapsulation of a cellular component, detailed information regarding the profile/kinetics (e.g., molecular weight, diffusion/mechanical properties, and degradation by-products) should be provided. In addition, most cell-scaffold TE/RM products cannot be terminally sterilized but use aseptic techniques and processes during manufacturing of the product; therefore information regarding scaffold sterilization methods used and stability/shelf-life are also critical. The flowchart shown in Fig. 84.2 provides the summary of some of the main characterization and safety considerations for each of the two main components, cell and scaffold, individually and in combination. It is also important to keep in mind that, oftentimes, the assembled product may be an intermediate form since final remodeling may occur in vivo, and/or maturation or preconditioning in a particular bioreactor is needed to achieve intended functions of the tissue construct prior to administration to patients. When in vivo remodeling, including scaffold degradation, is expected, the product may require extensive preclinical testing using adequate in vivo and in vitro models to assess the safety and performance of the final construct for potential construct failure prior to initiating a clinical trial. When maturation of an engineered tissue construct takes place in a bioreactor, it becomes important to evaluate information regarding material compatibility (including potential extractables and leachables), sterilization method (including sterilant residual), stability of construct in the bioreactor, characterization of the construct before and after bioreactor preconditioning/maturation, and sterility of the preconditioned/matured final construct. In some circumstances, the final product is not available for any destructive testing. In such cases, surrogate samples may be used in place of the final product if manufacturers provide sufficient information regarding how the surrogate samples are manufactured and provide supporting information and/or data to demonstrate the surrogates are representative of the final product.

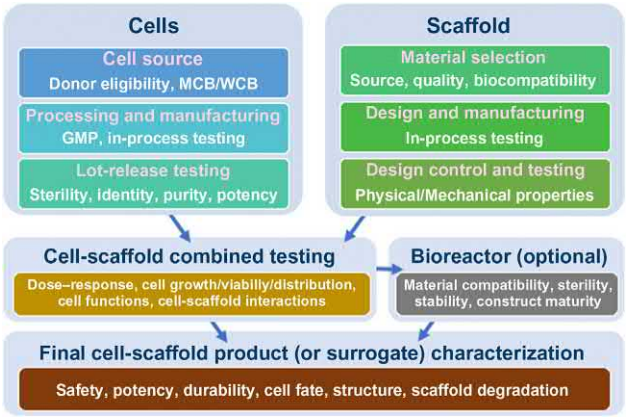

FIGURE 84.2 Safety and effectiveness consideration for a cell-scaffold TE/RM product. *GMP*, Good manufacturing practice; *MCB*, master cell bank; *TE/RM*, tissue-engineered/regenerative medicine; *WCB*, working cell bank.

A common misconception when developing tissue-engineered products is the importance of using "FDA-approved biomaterials." This misunderstanding may arise because certain types of biomaterials have been commonly used in TE/RM applications. In addition, FDA may take into consideration published scientific literature (when sufficient physicochemical data exist to demonstrate the similarity of previous and proposed biomaterials) as well as sponsor-derived test data to determine the safety of a biomaterial in a proposed product. However, it should be emphasized that FDA does not "approve biomaterials." This is because the clinical behavior and safety profile of a material can be profoundly affected by other factors, including the biomaterial source, manufacturing process, other components in the final product, anatomic location of the implantation site, and intended biological/physiological function of the final construct. When weighing the value of previously published data (and/or the need for additional studies), one should consider the intended use, source, manufacturing process, route of administration, and composition of the previous product.

3D bio-printed tissue-engineered/regenerative-medicine products

3D printing, including bioprinting, is rapidly emerging due to its potential to produce new medical products with unprecedented structural and functional designs. Bioprinting is increasingly used to generate TE/RM products by printing biological materials that include cells, biomaterials, and biomolecules. As described in an article by Ricles et al., differences in manufacturing methods between 3D printing and traditional manufacturing approaches have added specific technical considerations for 3D-printed products into the FDA scientific evaluation [46]. Because of the increased complexity of biological products, some additional technical considerations such as the printing parameters and consistency, material selection, finishing steps, vascularization of the construct, biocompatibility, mechanical and physicochemical properties, and biological function of the finished product may need to be considered.

Medical devices

A medical device is defined in Section 201(h) of the Federal Food, Drug, and Cosmetic Act (FDCA) in part as an instrument, apparatus, implant, etc., that is intended (1) for use in the diagnosis or other conditions; (2) for use in the cure, mitigation, treatment, or prevention of disease; or (3) to affect the structure or any function of man or other animals and which does not achieve its primary intended purposes through chemical action and is not dependent upon being metabolized for the achievement of its primary intended purposes. An investigational device exemption (IDE) application is typically required for a clinical investigation of an investigational product that meets the legal definition of a significant risk medical device or of a combination product in which the device component is thought to be providing the PMOA [40,47]. Information about the required contents of an IDE can be found in 21 CFR 812.20.

A risk-based approach determines the level of information for premarket review of medical devices. A premarket notification [510(k)] application seeks to demonstrate that a moderate risk medical device is substantially equivalent to another device already being marketed within the United States by presenting data on device design, bench testing and possibly clinical studies (21 CFR Part 807) [48]. For high-risk devices, including life-sustaining or life-supporting device, sponsors submit a PMA application that describes device composition and manufacture as well as the results of preclinical and clinical studies (21 CFR Part 814) [49,50]. Some of these devices are approved under Humanitarian Device Exemption applications, because these medical devices were designated as Humanitarian Use Devices which are intended to benefit patients in the treatment or diagnosis of a disease or condition that affects or is manifested in not more than 8000 individuals in the United States per year [defined by 21 CFR 814.3(n) and updated by the 21st Century Cures Act].

Least burdensome principles

Since the Food and Drug Administration Modernization Act of 1997 (FDAMA), Congress has directed FDA to take a least burdensome approach to medical device premarket evaluation in a manner that eliminates unnecessary burdens that may delay the marketing of beneficial new products, while maintaining the statutory requirements for clearance and approval. Accordingly, FDA issued the least burdensome guidance document, "The least burdensome provisions: concept and principles 2019," after the enactment of FDAMA [51]. Based on the least burdensome principle, the term "least burdensome" is the minimum amount of information necessary to adequately address a relevant regulatory question or issue through the most efficient manner at the right time. The guidance document stated that while the least burdensome provisions from FDAMA applied to PMA and 510(k) submissions, FDA believes that the least burdensome principles should be implemented for all medical device premarket regulatory activities.

Breakthrough device program

The 21st Century Cures Act provides new authority, the Breakthrough Devices Program, to help speed the review of certain innovative medical devices [52,53]. The Breakthrough Devices Program is a voluntary program for certain medical devices and device-led combination products that provide for more effective treatment or diagnosis of life-threatening or irreversibly debilitating diseases or conditions. The goal of the Breakthrough Devices Program is to provide patients and health care providers with timely access to these medical devices by speeding up their development, assessment, and review, while preserving the statutory standards for premarket approval, 510(k) clearance, and De Novo marketing authorization. The Breakthrough Devices Program offers manufacturers an opportunity to interact with the FDA's experts through several different program options to efficiently address topics as they arise during the premarket review phase, which can help manufacturers receive feedback from the FDA and identify areas of agreement in a timely way. Manufacturers can also expect prioritized review of their submission.

Evaluation of devices used with regenerative medicine advanced therapy

Section 3034 of the 21st Century Cures Act mandates that FDA issue guidance document clarifying how FDA will evaluate devices used in the recovery, isolation, or delivery of RMAT [3]. Although Section 3034 of the Cures Act refers to "recovery, isolation, or delivery," these terms are not defined in the Cures Act. Accordingly, FDA issued the guidance document "Evaluation of devices used with RMAT" to define the terms and to address how FDA intends to simplify and streamline its application of regulatory requirements for devices used to make combination device and cell or tissue products.

In the guidance document, the terms "recovery," "isolation," and "delivery" have the following definitions:

- *Recovery* means obtaining cells or tissues from a human donor.
- *Isolation* is processing that results in selection, separation, enrichment, or depletion of recovered cells or tissues that will become components of the final product.
- *Delivery* refers to any method by which an RMAT is introduced onto or into the body of a human recipient, for example, infusion, injection, topical application, or inhalation.

FDA recognizes that a wide range of devices may be used in conjunction with an RMAT. For example, the devices can be simple, low-risk devices, such as a manual surgical instrument (e.g., scalpel) for recovering cells and tissue. Devices used with RMATs may also be complex, higher risk devices, such as an automated cell collection system that selects and processes specific cells intended for an immediate return to the patient. In addition, devices can be constituent parts of an RMAT that is a combination product. It is noted that FDA does not consider scaffold devices combined with a cellular product to be within the scope of this guidance document. While such constructs may be eligible for RMAT designation, the scaffold constituent part would generally be considered a part of the RMAT and would generally not be considered solely a "device used in the delivery of an RMAT" because such scaffolds provide more than a delivery function.

Expedited review programs

There are several programs to facilitate and expedite the development and review of therapies that address unmet medical needs for the treatment of serious or life-threatening conditions [2]. These include four designation programs and one approval pathway: fast track designation, breakthrough therapy designation, RMAT designation, accelerated approval, and Priority Review (Table 84.1 and 84.2). CMC requirements for BLA submission remain the same even if the clinical testing is performed in the context of an expedited review program. The designation programs, particularly Breakthrough Therapy and RMAT designations, call for earlier attention from the FDA to these potentially promising therapies, offering sponsors earlier and more frequent interactions to receive FDA input on efficient trial design and overall drug development.

Fast track designation was established under the FDAMA to expedite the development and review of a new therapy to treat serious or life-threatening conditions and address an unmet medical need. The information necessary to demonstrate unmet medical need varies with the stage of drug development. Nonclinical or pharmacologic data will suffice in early-stage development while clinical data should be utilized in more advanced stages. If there

TABLE 84.1 Comparison of expedited review programs: criteria.

FT	BT	RMAT	AA	PR
• Serious condition **AND** • Nonclinical or clinical data demonstrate the potential to address unmet medical need	• Serious condition **AND** • Preliminary clinical evidence indicates that the drug may demonstrate substantial improvement over available therapy on one or more clinically significant endpoints	• Serious condition **AND** • It is a regenerative medicine therapy • Preliminary clinical evidence indicates that the drug has the potential to address unmet medical needs for such disease or condition	• Serious condition **AND** • Meaningful advantage over available therapies • Demonstrates an effect on either: a surrogate endpoint or an intermediate clinical endpoint	• Serious condition **AND** • Demonstrates potential to be a significant improvement in safety or effectiveness

Note: Information to demonstrate potential depends upon stage of development at which FT is requested. *AA*, Accelerated approval; *BT*, breakthrough therapy; *FT*, fast track; *PR*, priority review; *RMAT*, regenerative medicine advanced therapy.

Source: Modified from <https://www.fda.gov/downloads/Drugs/Guidances/UCM358301.pdf>.

TABLE 84.2 Comparison of expedited review programs: Features.

FT	BT	RMAT	AA	PR

• Frequent meetings
• Frequent written communication
• Eligibility for[a]
  ◦ *AA*
  ◦ *Privity review*
• Rolling review
	All of FT features
	AND
	• Intensive guidance on an efficient drug development program, beginning as early as Phase 1
	• Organizational commitment involving senior managers
		All of FT and ST features
		AND
		• Intensive guidance on an efficient drug development program, beginning as early as Phase 1
		• Organizational commitment involving senior managers
		• Statute addresses potential ways to support AA
			Approval based on surrogate *or* intermediate clinical endpoints
			• Save valuable time in the drug approval process
			• Reduce waiting period for patients to obtain clinically meaningful benefit
				• Short review clock
				• FDA will take action on an application within 6 months (compared to 10 months under standard review)

AA, Accelerated approval; *BT*, breakthrough therapy; *FDA*, US Food and Drug Administration; *FT*, fast track; *PR*, priority review; *RMAT*, regenerative medicine advanced therapy.

[a]*If relevant criteria are met.*
Source: Modified from <https://www.fda.gov.downloads/Drugs/Guidances/UCM358301.pdf>.

are existing therapies, a fast track eligible therapeutic must show some advantage over available treatment. A therapy that receives fast track designation is eligible for more frequent interactions with the FDA, rolling review, and accelerated approval or priority review if the requisite criteria are met.

Breakthrough therapy designation is a program that is intended to expedite the development and review of drugs for serious conditions when preliminary clinical evidence demonstrates that the product has the potential to provide a significant improvement in safety or effectiveness over existing therapies. This designation conveys the major advantages of the fast track program (rolling and priority review) as well as early intensive FDA guidance on efficient product development and commitment from senior managers.

The RMAT designation program is the newest program that was enacted in December 2016 pursuant to the 21st Century Cures Act to facilitate the development of RMTs. RMTs to treat, modify, reverse, or cure serious conditions are eligible for this program. The advantages of the RMAT designation include all the benefits of the fast track and breakthrough designation programs, including early interactions with the FDA to discuss potential surrogate or intermediate endpoints to support accelerated approval. In addition, the statute addresses potential ways to satisfy postapproval requirements.

Accelerated approval allows for the approval of a product for the treatment of a serious condition that has meaningful advantage over available therapies based on an effect on either a surrogate endpoint or intermediate clinical endpoint, with the requirement of confirmatory clinical data. Priority review allows for a shortened review clock of 6 months for products intended to treat serious conditions and, if approved, would provide a significant improvement in safety or effectiveness over available therapies.

Other regulatory topics

Minimal manipulation and homologous use of human cells, tissues, and cellular and tissue-based products

Due to the unique nature of HCT/Ps, the FDA implemented a tiered, risk-based approach to the regulation of HCT/Ps. Under these regulations, referred to as the "tissue rules" in 21 CFR part 1271, those HCT/Ps that meet all of the criteria in 21 CFR 1271.10(a) or fall within detailed exceptions (21 CFR 1271.15) do not require premarket review and approval. HCT/Ps that do not meet all of the criteria in 21 CFR 1271.10(a) and do not fall within detailed exceptions will be regulated as drugs, devices, and/or biological products and require premarket review and approval under the FDCA and/or Section 351 of the PHS Act.

Minimal manipulation

One of the four criteria for determining how an HCT/P is appropriately regulated is whether the HCT/P is minimally manipulated [54]. As defined in 21 CFR 1271.3(f)(1), minimal manipulation for structural tissue means processing that does not alter the original relevant characteristics of the

tissue relating to the tissue's utility for reconstruction, repair, or replacement. As defined in 21 CFR 1271.3(f)(2) minimal manipulation for cells or nonstructural tissues means processing that does not alter the relevant biological characteristics of cells or tissues. For applying the HCT/P regulatory framework, HCT/Ps that provide physical support or serve as a barrier or conduit, or connect, cover, or cushion are generally considered structural tissues, whereas HCT/Ps that function in metabolic or other biochemical capacities in the body are generally considered cells or nonstructural tissues. The determination of whether an HCT/P is minimally manipulated is dependent on the effect of manufacturing on the original relevant characteristics of the HCT/P as the HCT/P exists in the donor, not based on the intended use of the product in the recipient. As defined in 21 CFR Part 1271.3(ff), processing refers to "any activity performed on an HCT/P, other than recovery, donor screening, donor testing, storage, labeling, packaging, or distribution, such as testing for microorganisms, preparation, sterilization, steps to inactivate or remove adventitious agents, preservation for storage, and removal from storage." Other forms of processing also include cutting, grinding, shaping, culturing, enzymatic digestion, and decellularization.

Homologous use

A second criterion for determining how an HCT/P is appropriately regulated is the criterion of homologous use. As defined in 21 CFR 1271.3(c), homologous use means "the repair, reconstruction, replacement, or supplementation of a recipient's cells or tissues with an HCT/P that performs the same basic function or functions in the recipient as in the donor." FDA generally considers an HCT/P to be for homologous use when it is used to repair, reconstruct, replace, or supplement recipient's cells or tissues that are identical to the donor cells or tissues and perform one or more of the same basic functions in the recipient as the cells or tissues performed in the donor, or when used to repair, reconstruct, replace, or supplement recipient's cells or tissues that are not identical to the donor cells or tissues but that perform one or more of the same basic functions in the recipient as the cells or tissues performed in the donor. It is important to note that an HCT/P may still perform the same basic function or functions even when it is not used in the same anatomic location in the recipient as it existed in the donor.

Clinical research involving children

FDA regulations in 21 CFR Part 50 Subpart D outline special protections for children, which are in addition to the safeguards for all clinical trial subjects that are contained in 21 CFR 312. For clinical studies involving more than a minor increase over minimal risk, the additional safeguard under 21 CFR 50, Subpart D, and in particular 21 CFR 50.52 (i.e., such clinical investigations must offer the prospect of direct benefit to the pediatric subjects), is applied. In general, initial studies of cell-based products should be conducted in an adult population prior to investigations in a pediatric population to procure some preliminary safety- and activity-related information. However, in some situations, first-in-human studies may be ethical in a pediatric population, for instance, when the disease primarily affects children and the benefit/risk profile in adults may not be favorable.

For pediatric studies that involve more than minimal risk, the considerations for IRB approval are outlined in 21 CFR 50.52 [55].

Expanded access to investigational drugs for treatment use

FDA's expanded access regulations (21 CFR 312 Subpart I) allow access to investigational drugs for the primary purpose of diagnosing, monitoring, or treating a patient's disease or condition, rather than for generating scientific data intended to characterize the drug [56]. The intent of these regulations is to facilitate the availability of these products to patients with serious diseases or conditions when no comparable or satisfactory alternative therapy exists. The regulations describe the criteria that must be met to authorize expanded access.

Charging for investigational drugs under an investigational new drug application

The IND regulations allow sponsors to charge for administration of investigational products in clinical trials and to charge for investigational products made available under FDA's expanded access regulations (21 CFR 312.8) [57]. These regulations specify general criteria for authorizing charging, specific criteria for charging for an investigational drug in a clinical trial, and specific criteria for charging under the expanded access provisions and clarify what costs can be recovered.

Responsibilities of sponsors and investigators

Sponsors and investigators are responsible for specific activities to ensure that the rights, safety, and welfare of subjects are protected, to ensure the quality of the clinical trial, and to meet regulatory requirements (21 CFR 312 Subpart D). The FDA regulations (21 CFR 312.3) divide clinical trial responsibilities among these two parties: sponsors are responsible for communicating with FDA through the IND and managing the clinical trials; clinical

investigators conduct the studies and administer the investigational product to subjects.

Sponsor responsibilities (21 CFR 312.50–59)

Sponsors have several responsibilities for managing the clinical trials under an IND. Sponsors must select investigators who are qualified by training and experience to administer the investigational product to study subjects and conduct safety and efficacy monitoring. Each study site should have an investigator who signs Form FDA 1572, listing the name, address, and the locations where the study will be conducted, all subinvestigators, and the IRB responsible for review of the study (21 CFR Part 56). The investigator is not required to be a physician, but it is reasonable to assume that a physician will be part of the study team. The FDA expects that the sponsor will evaluate potential clinical sites to determine that the facilities and resources are adequate to conduct the study [58].

Sponsors must provide investigators with the protocol, the IB, and any other instructions necessary to conduct the study. As the study progresses, the sponsor must communicate any protocol revisions and new information about the safety of the investigational product to the investigators. Sponsors must also monitor the progress of all investigations conducted under the IND to ensure they are conducted in accordance with the general investigational plan and protocol [59]. If an investigator is not complying with the protocol, the sponsor must either obtain an assurance of compliance from the investigator or discontinue shipments of the product to the investigator. Particularly for cell and gene therapy products, including xenogeneic cellular products, the protocol may need to include procedures for long-term monitoring of subjects, in accordance with FDA and PHS regulations and PHS guidelines.

IND safety reporting is another sponsor responsibility (21 CFR Part 312.32). Sponsors must promptly (≤15 calendar days) notify FDA and all investigators receiving investigational product in an IND safety report of potential serious risks. Sponsors must also promptly (≤7 calendar days) notify FDA of any unexpected fatal or life-threatening suspected adverse reaction.

Sponsors must maintain adequate records of financial interest and records of the receipt, shipment, use, or other disposition of investigational product. All records related to the IND must be retained for 2 years after a marketing application is approved, or for 2 years after shipment and delivery of the investigational product was discontinued and FDA was notified. Sponsors must permit access for FDA to inspect the study records. A sponsor may transfer any or all of the sponsor's IND obligations to contractors, who then assume the obligations of the sponsor for the designated activities.

Clinical investigator responsibilities (21 CFR 312.60–69)

Clinical investigators are responsible for protecting the rights, safety, and welfare of the subjects under the investigator's care. Investigators are required to conduct the study in accordance with the protocol, for example ensuring that subjects meet the eligibility criteria and that all follow-up assessments are performed as scheduled. They must personally supervise the administration of the investigational product and the conduct of the study. The investigator may delegate certain duties to the subinvestigators that are under his/her supervision, but he/she cannot transfer his/her regulatory obligations to others. Investigators must obtain the review of the study by the IRB that oversees their clinical site and must obtain the informed consent of study subjects prior to enrolling them into a study. The investigator shall also notify the IRB (21 CFR 312.66) of all unanticipated problems involving risk to human subjects or others.

Investigators must limit the use of the investigational product to only the subjects enrolled in the study and must maintain records of the storage and use of the product. Investigators must report AEs and progress reports to the IRB and to the sponsor so both are aware of new information about the safety of the investigational product. Investigators must also submit financial disclosure forms to the sponsor and update them when required. Investigators must prepare and maintain adequate and accurate case histories for each subject and must retain all study records for 2 years after a marketing application is approved, or for 2 years after the investigation is discontinued and FDA is notified. Investigators should plan for how they will maintain these records for several years, especially if they are participating in early-phase studies. When the study at a clinical site has been completed, the investigator must submit a final report to the sponsor and must allow access for FDA to inspect the study records.

Sponsor–investigator responsibilities

A sponsor–investigator is an individual who is the IND sponsor and is the only clinical investigator for a clinical study of the product he/she is developing. Sponsor–investigators are required to fulfill all the responsibilities of sponsors and of investigators, except for the requirement for an IB. Sponsor–investigators must consider how to monitor the clinical trial he/she is personally conducting. Some options include the review of the study by someone unaffiliated with the research, such as a colleague in another department, from an institution's research office, or by a contract research organization.

Clinical research conducted outside of the United States

A sponsor who is conducting a foreign clinical study may choose, but is not required, to conduct the foreign clinical study under an IND. When a foreign clinical study is conducted under an IND, all FDA IND requirements must be met unless a waiver is obtained. FDA may accept a well-designed, well-conducted, non-IND foreign study as support for an IND or application for marketing approval if the study was conducted in accordance with good clinical practice and if FDA is able to validate the data from the study through an onsite inspection, if necessary (21 CFR 312.120). Regarding the ability of data from a non-IND foreign study to support a marketing application (21 CFR 314.106), an important consideration is the applicability of the data to the US population [60].

Use of standards

The 21st Century Cures Act, Title 3 Section 3036 directs FDA, in consultation with the National Institutes of Standards and Technology (NIST) and stakeholders to "facilitate an effort to coordinate and prioritize the development of standards and consensus definition of terms, through a public process, to support, through regulatory predictability, the development, evaluation, and review of regenerative medicine therapies and regenerative advanced therapies, including with respect to the manufacturing processes and controls of such products." [1]

The scientific and manufacturing novelty of many RMTs including TE/RM products, present unique challenges for meeting regulatory requirements with respect to product testing, performance characteristics, testing methodologies, scientific protocols, product quality, ingredient specifications, and compliance criteria. Increased development and use of standards have the potential to transform product development, contribute to regulatory predictability, and facilitate the overall development of RMTs [42,44,61,62].

US Food and Drug Administration international regulatory activities

With the globalization of medical products, especially RMTs, OTAT is leading several initiatives that help facilitate FDA's goals to safeguard global public health and enable the availability of safe and effective products [63]. The International Pharmaceutical Regulators Program (IPRP) Cell Therapy Working Group (CTWG) is a forum for regulatory bodies that allows for open discussion and sharing of best practices for the regulation of cell and tissue-based products. The scope of products covered in the CTWG includes cell-therapy products without gene modifications, tissue-engineered products, and xenotransplantation products. Discussions are focused on topics that may be suitable for regulatory convergence, and producing publicly available position papers and to support harmonization initiatives such as the Asia Pacific Economic Cooperation (APEC) and Pan American Health Network for Drug Regulatory Harmonization (PANDRH), International Council of Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), Pharmaceutical Inspection Convention and Pharmaceutical Inspection Cooperation Scheme (PIC/S), and World Health Organization (WHO).

OTAT is also focused on activities that foster the independent development of national guidelines and regulations that reflect a shared regulatory perspective internationally. International activities for RMTs range from formal arrangements with international regulatory authorities to ad hoc product-specific discussion with international regulatory partners. Examples of formal activities include "Cluster" meetings on various topics such as the advanced therapy medicinal product cluster with the European Medicines Agency (EMA), Health Canada, and Japan Pharmaceuticals and Device Agency to assist sponsors and product developers in making submissions to the FDA and EMA for a specific product, sponsors and developers can request Parallel Scientific Advice or Consultative Advice. These processes are outlined in "General Principles EMA-FDA Parallel Scientific Advice (Human Medicinal Products)" [64].

The role of cell-based products in medical product testing

A major priority of the FDA's strategic plan for advancing regulatory science includes efforts to modernize toxicology to enhance product safety. This emphasis recognizes the need to improve the predictive value of preclinical studies for all medical products, and the potential role for cell and tissue-based assays (a subset of bioengineered tissue products) in achieving this goal [65]. Implicit in this goal is the need to improve manufacturing processes, to increase the understanding of the biology, and clinical applicability of cell and tissue-based assays.

Conclusion

Federal regulations address many aspects of drug development, including CMC, preclinical development, and clinical trials. Efficient drug development depends on an understanding of these regulations and the regulatory process. OTAT collaborates with sponsors to address the challenges that arise during the development of cell-based products [66].

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Appendix I Code of Federal Regulations citations relevant to cellular product development[4]

CFR citation	Summary of regulation
9 CFR 113.53	Requirements for ingredients of animal origin used for production of biologics
21 CFR Part 3	Product jurisdiction
21 CFR Part 50	Protection of human subjects
21 CFR Part 50 Subpart B	Informed consent (IC) of human subjects
21 CFR Part 50 Subpart D	Additional safeguards for children in clinical investigations
21 CFR Part 56	Institutional review boards (IRB)
21 CFR Part 58	Good laboratory practice (GLP) for nonclinical laboratory studies
21 CFR Parts 210 and 211	Current good manufacturing practice (cGMP) for finished pharmaceuticals
21 CFR Part 312	Investigational new drug application (IND)
21 CFR Part 312.8	Charging for investigational drugs under an IND
21 CFR Part 312.23	IND content and format
21 CFR Part 312.32	IND safety reporting
21 CFR Part 312.42	Clinical holds and requests for modification
21 CFR Part 312.50–59	Responsibilities of sponsors
21 CFR Part 312.60–69	Responsibilities of investigators
21 CFR Part 312.106	Foreign clinical data in a marketing application
21 CFR Park 312.120	Foreign clinical studies not conducted under an IND
21 CFR Part 312 Subpart I	Expanded access to investigational drugs for treatment use
21 CFR Part 600	Biological products
21 CFR Part 600.80	Postmarketing reporting of adverse experiences
21 CFR Part 601	Biologics licensing
21 CFR Part 601.2	Applications for biologics licenses; procedures for filing
21 CFR Part 610	General biological products standards
21 CFR Part 610.12	Sterility
21 CFR Part 800	General medical devices
21 CFR Part 801 or 809	Labeling
21 CFR Part 812	Investigational device exemptions (IDEs)
21 CFR Part 807	Establishment registration and device listing for manufacturers and initial importers of devices
21 CFR Part 814	Premarket approval (PMA) of medical devices
21 CFR Part 814 Subpart H	Humanitarian Use Devices (HDE)
21 CFR Part 820	Quality system regulation (QSR)
21 CFR Part 1271	Human cells, tissues and cellular and tissue-based products (HCT/Ps)

Appendix II The list of acronyms

FDA	**US Food and Drug Administration**
CDER	Center for Drug Evaluation and Research
CBER	Center for Biologics Evaluation and Research
CDRH	Center for Devices and Radiological Health
OTAT	Office of Tissues and Advanced Therapies
RMTs	Regenerative medicine therapies
RMAT	Regenerative medicine advanced therapy
CFR	Code of Federal Regulations
PHS	Public Health Service
IND	Investigational new drug application
BLA	Biologics licensing application
POC	Proof-of-concept
CMC	Chemistry, manufacturing, and controls
MCB	Master cell bank
WCB	Working cell bank
AE	Adverse event
INTERACT	Initial Targeted Engagement for Regulatory Advice
OCOD	Office of Communication, Outreach and Development
IRB	Institutional review boards
IB	Investigator's Brochure
eCTD	Electronic common technical document
DS	Drug substance
DP	Drug product
cGMP	Current good manufacturing practice
OCBQ	Office of Compliance and Biologics Quality
DMPQ	Division of Manufacturing and Product Quality
SPA	Special Protocol Assessment
PMOA	Primary mode of action
PMA	Premarket application
TE/RM	Tissue-engineered and regenerative medicine
IDE	Investigational device exemption
FDCA	Federal Food, Drug, and Cosmetic Act
FDAMA	Food and Drug Administration Modernization Act of 1997
HCT/P	Human cells, tissues, and cellular and tissue-based products
GCP	Good Clinical Practice
NIST	National Institutes of Standards and Technology
IPRP	International Pharmaceutical Regulators Program
CTWG	Cell Therapy Working Group
APEC	Asia Pacific Economic Cooperation
PANDRH	Pan American Health Network for Drug Regulatory Harmonization
ICH	International Council of Harmonization of Technical Requirements for Pharmaceuticals for Human Use
PIC/S	Pharmaceutical Inspection Convention and Pharmaceutical Inspection Cooperation Scheme
WHO	World Health Organization
EMA	European Medicines Agency

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Chapter 85

Business issues

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Introduction

Imagine a world of medicine where replacement or repair of body parts could be accomplished using tissues grown in a laboratory. Or better yet, the body could be induced to repair and regenerate itself—to harness the body's natural healing processes and activate these processes when and where they are critically needed. Aubry de Grey, a biomedical gerontologist whose research focuses on how regenerative medicine can prevent the aging process, has famously stated that "The First Person to Live to 150 has Already Been Born"; a headline which could all too easily be dismissed as being overly boastful. The question to ask ourselves, however, is should we be so quick to write off such a possibility? Whether or not the first sesquicentenarian is already among us aside, the progress in preclinical and clinical regenerative medicine programs, particularly stem cells, has picked up considerable pace and is being continually primed by a growing pipeline of promising research and therapeutic programs aimed at repair and regeneration. In the last 120 years, as a consequence of lifestyle and nonregenerative advances in medicine, we have seen an increase in average life expectancy from 43 to 85 years. As we achieve the ability to extend or replace tissues and organs—to begin to realize the aspirational goals of tissue engineering, and regenerative medicine in general—would doubling life expectancy over the next 150 years period in fact be a possibility? And if so, what are the societal and commercial implications?

The past 30 or so years have seen the emergence of an area of medicine called tissue engineering and regenerative medicine. This discipline is a truly multidisciplinary field, involving scientists, engineers, and physicians working to construct biological substitutes which can replace (or help regenerate) diseased and injured tissues. It promises to revolutionize the ways we approach health and quality of life for tens of millions of people worldwide. The field has already produced notable examples of actual therapies, including skin replacement and cartilage repair. The pump is primed, but companies, payers, and policy makers will need to assess long-range cost benefit trade-offs for engineered tissues and cell therapies when compared to traditional therapies. Understanding the interplay giving rise to opposing pressures—one from an expanding elderly population to improve health in old age, and the other a growing concern for containing health-care costs—will be critical to tissue and cell therapy companies as they plan for and address concerns about how patients can afford innovation in this important area of treatment.

The aging population

To consider the implications that further enhancing longevity through tissue engineering may have on society and medicine, one needs to start by understanding current trends and projected impacts. The age structure of the overall population is projected to change greatly over the next 40 years. The world's population is aging, primarily as a function of declining fertility coupled with increasing life expectancy. Aging is occurring not only in high-income countries but also in middle- and low-income countries. In fact, between now and 2050, the world is projected to experience rapid growth in its older population. Fig. 85.1 shows the projected growth of two groups of older people—those aged 65−79 and those 80 years and older. The rapid growth of the over-80 group is related to increases in life expectancy related to improving medical care and nutrition, which have occurred during the century.

People live longer now than at any time in the past; with advances in medicine promising to extend life expectancy even further. The US life expectancy at birth rose from 47.3 years in 1900 to 76.9 years in 2000. Greater longevity, combined with relatively low fertility rates, has rapidly increased the proportion of the oldest old among

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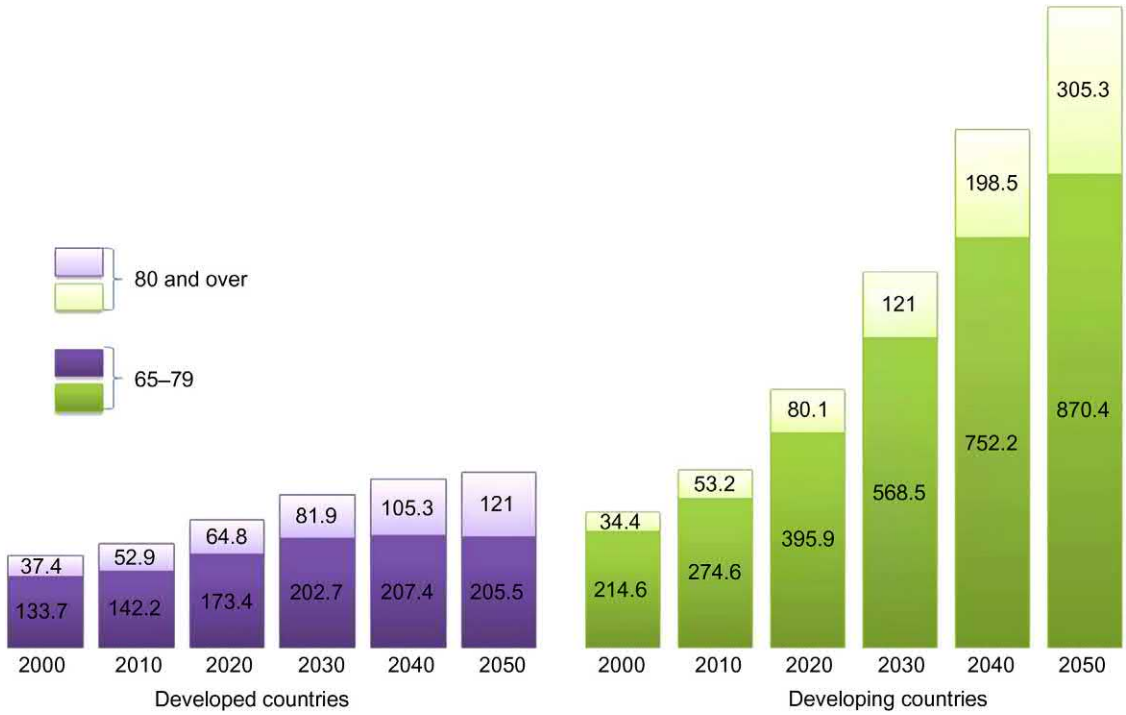

FIGURE 85.1 Population aged 65 and over for developed and developing countries (in millions). *U.S. Cenus Bureau Publication. 65 + in the United States. p. 23–209.*

the total older population. In 1900 only 4.0% of all older people were aged 85 and older; by 2000 that proportion had grown to 12.1% [1].

In 2050 the number of people over the age of 65 living in (now) developed countries is projected to exceed 325 million, with more than 1.5 billion persons in this over-65 category when including the developing countries in the equation—a nearly tripling of the number of over-65 people living in all these countries today. In the United States, as an example, the number of Americans aged 65 and older is projected to be 88.5 million, more than double the population of 40.2 million in 2010. The baby boomers are largely responsible for this increase in the older population, as they recently began crossing into this category in 2011. By 2050 the US population is projected to grow by 439 million, an increase of 42% relative to the 2010 census numbers of 310 million. The nation will also become more racially and ethnically diverse. The population is also expected to become much older, increasing from 15% today to nearly 20% of the US population being aged 65 and older by 2030, and continuing to increase to about 22% by 2050. Those over the age of 85 accounted for 5.8 million Americans in the 2010 census and are expected to reach 8.7 million by 2030 and then 19 million by 2050. In terms of overall population, this "oldest" old population will increase to 4.3% of the population by 2050—representing only 1.8% of the population in the 2010 census.

Much of the advances in longevity early in the 20th century arose from improvements in socioeconomic and living conditions and a decrease in infectious disease deaths. Gains during the latter part of that century came from periodic breakthroughs in public health and biomedical research that have led to new treatments for, and a later onset of, chronic diseases [2]. If improvements in treating and even preventing the onset of chronic diseases can be sustained and further enhanced, then the age structure of the older population will be even more positively affected than the previous projections suggest. This aging of the population will have wide-ranging implications. The projected growth of an older population will present challenges to policy makers and programs, such as Social Security and Medicare in the United States. The health status of the aging population is essential not only to those who comprise this age group but also to the broader population because of the impacts on social and economic systems. As the older population grows not only in size but more importantly in their proportion of the total population, the potential implications for families, businesses, and health-care providers will become increasingly significant.

Chronic diseases and impairments, which are among the leading causes of disability in older people, can negatively impact quality of life, lead to a decline in independent living, and impose an economic burden. About 80% of seniors have at least one chronic health condition and

50% have at least two [3]. Indeed, of the roughly 150,000 people who die each day, about two-thirds die of age-related causes. In industrialized nations the proportion is much higher, reaching 90% [4].

Concern is growing that medical advances leading to longevity will, in turn, lead to an older population who have a higher incidence of functional and cognitive impairment. With increases in life expectancy and a simultaneous rise in the number of people with chronic diseases and disability, researchers are focusing on facilitating both longer life and disability-free healthy life. Focusing on the quality as well as length of life, the World Health Organization has introduced estimates of "healthy life expectancy" (HALE), which is defined as the average number of years of life free from disability, physical performance limitations or impairments, other disabilities, or social handicaps [5]. Using various measurements and methods of analysis, including HALE, recent studies conclude that in addition to living longer, the current generation of older people is healthier and less disabled than the preceding ones [6–8]. But Fig. 85.2 illustrates, as life expectancy increases so does the incidence rate of chronic and degenerative health conditions, threatening to create a wider gap between HALE and total life expectancy.

Currently, the vast majority of treatments for chronic and/or life-threatening diseases are palliative. Others delay disease progression and the onset of complications associated with the underlying illness. Only a very limited number of therapies available today are capable of curing or significantly changing the course of disease. The result is a health-care system burdened by costly treatments for an aging, increasingly ailing population, with few solutions for containing rising costs. Fig. 85.3 demonstrates the predicted impact that our aging population is expected to have on the cost of healthcare in less than 20 years time. The demographics shift in aged populations toward older adults in the United States alone will likely add more than one trillion dollars to the direct cost of healthcare, and multiples of that amount for the indirect costs associated with accommodating the needs of older Americans. According to a 2017 report from the National Health Expenditure Accounts, health-care expenditures in the United States are currently about 18% of GDP, and this share is projected to rise sharply. If health-care costs continue to grow at historical rates, the share of GDP devoted to healthcare in the United States is projected to reach 34% by 2040. Viewing the distribution of the cost across all working Americans, Fig. 85.4 shows the potential impact on total compensation when the projected rising cost of healthcare is factored into predicted salaries.

Rise of regenerative medicine

The best way to address the escalating economics of healthcare includes developing more effective treatments, and even cures, for the most burdensome diseases—diabetes, neurodegenerative disorders, stroke, macular degeneration, and cardiovascular disease, for example—to facilitate longer, healthier, and more productive lives. Regenerative technologies, such as cell- and tissue-based therapies, have the power to be a critical component in reducing the otherwise predicted increase in health-care costs resulting from aging. A more effective, sustainable health-care system is a potential as a consequence of the innovations occurring in regenerative medicine. However, it will require the concerted efforts of patients, government and private insurance companies, doctors, life science companies, private investors, and governments. Regenerative medicine promises to completely change the way we think about disease, aging,

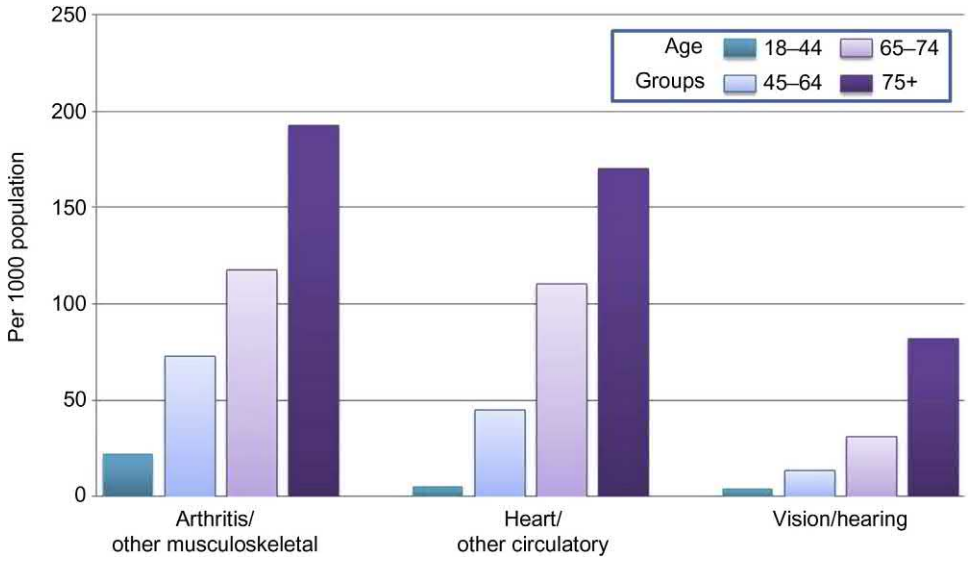

FIGURE 85.2 Exemplary chronic health conditions. *U.S. Cenus Bureau Publication. 65+ in the United States. p. 23–209.*

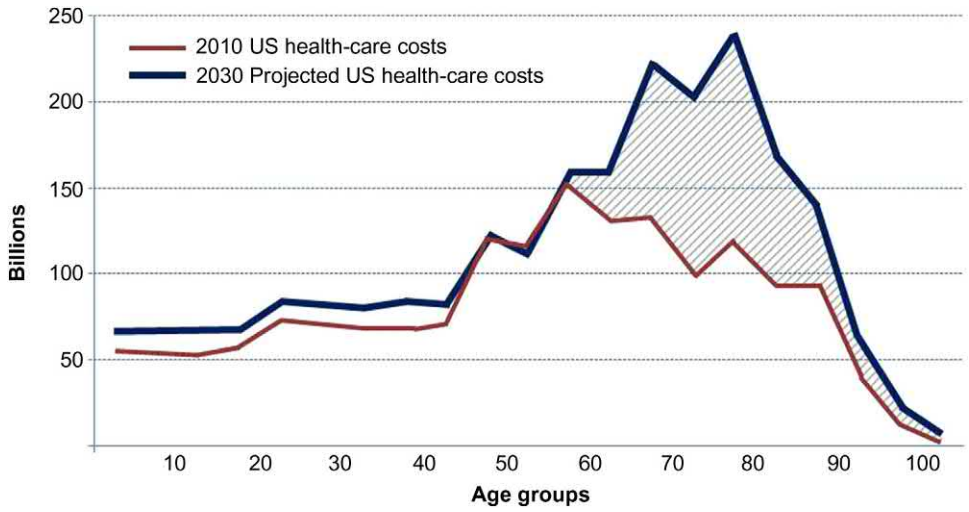

FIGURE 85.3 Impact of aging on the US health-care costs. *Alliance for Regenerative Medicine. 2012 annual industry report.*

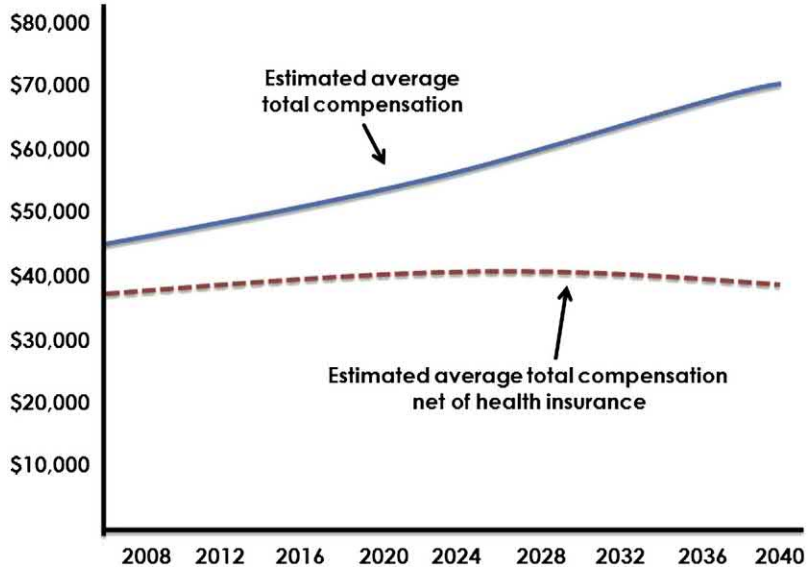

FIGURE 85.4 Projected annual compensation and compensation less health insurance premiums. *Office of the President, Council of Economic Advisors. The economic case for healthcare reform; June 2009.*

and even the practice of medicine itself—and, as the promise goes, to help bend down the ascending curve of projected health-care costs.

Regenerative medicine, and particularly cell and tissue therapies, has already had some clinical impacts. More than one million patients worldwide have already had bone marrow or hematopoietic stem cell transplants, with more than 50,000 new transplants being carried out each year [9,10]. A more recent category of approved products includes tissue-engineered skin, with more than 250,000 patients having now received artificial skin grafts [11]. In parallel, treatments for oncological and inflammatory diseases now include adoptive cell transfer and engineered immune cells, such as CAR-T, CAR-NK, and CAR-Treg cells. While the number of patients treated with cell therapies is still low by the standards of conventional drug- or biologics-based treatments, there is a robust pipeline of new therapies in clinical or preclinical testing.

However, the realization of the power of cell therapies, and with it the emergence of this growing area of medicine, is not only dependent on resolving the science but also on a number of business factors unique to cells as therapeutic agents. Regulatory oversight, clinical endpoints, drug labeling, pricing and reimbursement, manufacturing, cold chain, and essential infrastructure are some of the many components from the business perspective which typically have unique and far-reaching consequences in the path from the laboratory bench to patient treatment. Laminated onto these business issues can be layers of political, legal, and ethical sensitivities which need to be considered. Regenerative medicine is uniquely capable of altering the fundamental mechanisms of disease; however, to realize its potential,

we must think differently about therapeutic development and commit to investing in these transformative technologies. A more effective, sustainable health-care system is possible through regenerative medicine, but it will require the combined efforts of patients, payers, health-care providers, biotech and pharmaceutical companies, private investors, and governments working together.

The reality is that not every new company or technology in this space can or will succeed. Not every new invention relating to regenerative medicine is necessarily commercially tractable. The technology may be too complex or high touch to be used in an FDA-regulated commercial setting, or too costly, particularly where reimbursement by insurance entities or the government needs to be considered. Unfortunately, great science does not always translate into great business. The history of the life science industry is littered with many examples of brilliant research and breakthroughs unable to be translated into a viable commercial product. Understanding what are often the gating issues and how to manage through those issues, including recognizing the "go/no go" decision points, can greatly increase the likelihood of success. Realizing the promise of regenerative medicine from the perspective of the life science company will require understanding the regulatory policies in the major markets, appreciating the impact of final manufacturing, final formulation and the cold chain of the distribution, as well as developing strategic business and reimbursement plans. Added to this equation for success is the inevitable complexity of intellectual property issues.

Product development

Tissue engineering encompasses an array of technologies in biology, chemistry, and physics into materials, devices, systems, and a variety of therapeutic approaches—including cell-based therapies—to augment, repair, replace, or regenerate organs and tissues (see Fig. 85.5), with the end goal often being curative by targeting the root cause of disease. This rapidly evolving, interdisciplinary field in healthcare is transforming the practice of medicine, medical innovation, and the production of medical devices and therapies.

Living cells are incorporated into regenerative medicines to achieve a variety of positive effects, such as replacing damaged or diseased cells and/or tissue; stimulating healing and regeneration in diseased tissue; and delivering small molecule therapies to targeted areas. However, by their very nature, cell and tissue therapies face fundamentally different development and regulatory pathways to market when compared to traditional small molecule-based drugs or protein therapeutics. Alongside the complexity of the agent being administered to patients come added layers of safety and efficacy concerns. Cell therapies face the challenge of manufacturing a living product alongside associated difficulties in their storage and delivery. Depending on the solutions, companies may need to decide whether to focus on manufacturing a product or becoming a service-based entity, each of which requires a fundamentally different business strategy. As of 2018 the regenerative medicine industry to include more than 900 companies ranging from divisions of global pharmaceutical companies to smaller life science companies focused on regenerative medicine technologies, including gene and cell therapies, and engineered tissue therapeutics [12]. The product pipeline of these groups includes hundreds of cell-based therapies, small molecules, biologics, tissue-engineered cells and materials, and implantable devices with more than 1000 clinical trials using regenerative medicine technologies [12]. Other companies work toward providing research tools such as equipment, consumables, software, cells as drug discovery or toxicity testing tools as well as clinical tools, bioprocessing tools, and platforms that include equipment, consumables, reagents, and storage systems. The field also incorporates a variety of service companies specializing in clinical trial management, manufacturing, engineering, and financing among others.

An important concern for emerging life science in the regenerative medicine field relates to developing a compelling business model. It is important to understand the consequences arising from, for example, the choice of technology by a company with how it relates to the practical realities of commercialization, particularly manufacturing, distribution, and adoption by health-care providers, not to mention coding and reimbursement. There are fundamental differences

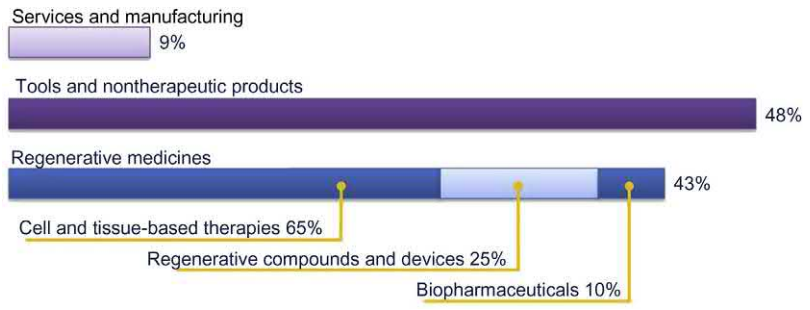

FIGURE 85.5 Regenerative medicine industry sectors. *Alliance for Regenerative Medicine. 2012 annual industry report.*

in the business models arising from the choice between an allogeneic cell or tissue product (a source which can be scaled-up and universally applied to patients) versus providing autologous cells or tissues requiring the manipulation of a patient's own tissues. The choice between the allogeneic and autologous therapy routes is proving to be a fundamental one for early-adopting companies with significantly different production, infrastructure, logistics, skills, and storage requirements for each approach. Autologous cell therapies already represent a significant industry, though principally as a service-based model rather than something more akin to a pharmaceutical or biologics business model. One advantage of autologous transplants is that the body recognizes the cells as self and therefore does not reject the transplanted materials. For example, bone marrow transplants have been successfully used for many years to restore immune function after chemotherapy, and therapeutic trials using patients' own stem cells to restore other tissues are already underway. The allogeneic cell transplants have greater potential for scale-up and widespread distribution, thus potentially benefiting from greater economies of scale. This model is closer to the traditional pharmaceutical model and shares many similarities to biomanufacturing processes that pharma have already invested in heavily over the last decade. However, allogeneic therapies face a greater risk of immunological rejection as the cells are typically not Human Leukocyte Antigens (HLA) matched to the intended recipient patient. For these reasons the allogeneic business model is anticipated to develop more slowly, beginning first with cells and tissues that are transplanted in immune-privileged sites in the body such as the Central Nervous System (CNS) or subretinal space, or for which the cell is itself immune-privileged, as is the case with mesenchymal stem cells.

In many respects, regenerative medicine companies are not harder to start up than other life science companies. Yet, establishing a successful cell therapy company poses some unique challenges, in part, because the field is only recently emerging and the regulatory oversight and reimbursement for patient therapy are still undefined in many ways. In the end, cell and tissue therapies are likely to be more expensive to develop than small molecule or protein therapeutics largely because the safety and proof-of-concept in relevant animals, if available at all, that the regulatory agencies want as part of the preclinical package. Yet, those risks can be dwarfed by the concern that potential therapies will get entangled in regulatory red tape before they can even work their way through clinical trials.

Embryonic stem cells

One of the fundamental issues arising from the use of adult-sourced human stem cells for allogeneic therapies or ex vivo engineered tissues derives from the limited replicative capacity that adult stem cells have in culture. Very often, the adult cells will undergo only a handful of cell doublings before large percentages of the cells in culture senesce or differentiate into unwanted cell types. In contrast, a human embryonic stem (ES) cell (hESC) line represents a nearly inexhaustible supply of pluripotent cells. Derived from a single clonal ES cell, the replicative capacity of an hESC line is enormous. However, for a long time, there was a concern in the industry around whether or not the regulatory agencies would permit a differentiated cell made from an hESC line to even be tested in patients. The concern, at least as it manifests itself in the industry and among potential investors, arose from the ability of ES cells to form any tissue in the body—including tumors. The fundamental question was under what circumstances would the FDA or EMA (for instance) permit a differentiated cell product to be tested in human patients? How would they control for the risk that a cell therapy product might be contaminated with an undifferentiated cell, particularly an ES cell?

That question was finally answered in 2010 when two companies, Advanced Cell Technology (now Astellas Institute for Regenerative Medicine) and Geron, obtained FDA to conduct Phase I/II studies in human patients using cells derived from hESC lines. While Geron ultimately abandoned its cell therapy programs to focus on oncology product lines, Advanced Cell Technology moved forward in clinical trials both in the United States and the United Kingdom for treating various forms of macular degeneration using retinal pigment epithelial (RPE) cells derived from an hESC line. In the company's IND and IMPD filings with the US and UK regulatory agencies, respectively, Advanced Cell Technology described a carefully controlled cell culture process that, when shifted from proliferation to differentiation, was incapable of supporting the growth of undifferentiated ES cells. Moreover, the company had developed a release assay for the final RPE cellular product that was several orders of magnitude more sensitive than Polymerase Chain Reaction (PCR) for detecting contamination with undifferentiated cells. Putting these two manufacturing steps together, along with safety data from hundreds of animals injected with the human RPE cells, the company was able to establish an acceptable reduced risk profile to be permitted to test the RPE cells in human patients.

In July 2011, Advanced Cell Technology treated its first two patients with hESC-derived RPE cells. One patient suffered from Stargardt's disease, a juvenile-onset form of macular degeneration, and the other patient suffered from dry age-related macular degeneration (AMD), the most prevalent form of AMD and the leading cause of blindness in people over the age of 65. In 2015 the company published its findings from its two prospective phase

I/II studies carried out in the United States intended to assess the primary endpoints safety and tolerability of subretinal transplantation of hESC-derived retinal pigment epithelium. The article reported among the 18 patients treated that there was no evidence of adverse proliferation, rejection, or serious ocular or systemic safety issues related to the transplanted tissue—and that the observed adverse events were associated with vitreoretinal surgery and immunosuppression. A total of 72% (13 of 18 patients) had patches of increasing subretinal pigmentation consistent with transplanted retinal pigment epithelium. Best-corrected visual acuity, monitored as part of the safety protocol, improved in the treated eye of 10 of the patients, improved or remained the same in 7 patients, and decreased in only 1 of the patients. In contrast the untreated fellow eyes (a pseudo same patient control) did not show similar improvements in visual acuity as observed in the treated. Vision-related quality-of-life measures increased for general and peripheral vision, and near and distance activities, improving by 16−25 points 3−12 months after transplantation in patients with atrophic AMD and 8−20 points in patients with Stargardt's macular dystrophy [13]. The interpretation of these results was that the studies provided the first evidence of the medium-term to long-term safety, graft survival, and possible biological activity of pluripotent stem cell progeny in individuals with disease, and in particular, it suggested that hESC-derived cells could provide a potentially safe new source of cells for the treatment of various unmet medical disorders requiring tissue repair or replacement.

Induced pluripotent stem cells

As described in greater detail in earlier chapters, terminally differentiated somatic cells can be reprogrammed to generate induced pluripotent stem cells (iPSCs) by the enforced expression of a few embryonic transcription factors. The resulting iPSCs are morphologically and phenotypically similar to ES cells and thus offer exciting possibilities in stem cell research and regenerative medicine. The first iPSCs were produced in 2006 from mouse cells and, subsequently, in 2007 from human cells [14,15]. The most widely used set of reprogramming factors, Oct4, Sox2, Klf4 and c-Myc, was identified initially by screening 24 preselected factors in mouse embryonic fibroblasts by Takahashi and Yamanaka. Forced expression of these factors initiates phenotypic and molecular changes in the targeted somatic cell, eventually leading to the reactivation of endogenous pluripotency genes and acquisition of pluripotency [16]. The ability to restore pluripotency to adult cells through the induction of a small number of reprogramming factors promises to create powerful new opportunities for modeling human diseases, and perhaps more excitingly, offers hope for patient-specific cell therapies.

However, the challenge now is to better define differences in the epigenetics and gene expression of the resulting cells and subsequently improve the reprogramming methods in order to make human iPSCs a truly tractable alternative to hESCs. While at first approximation ES cells and iPSCs appeared equivalent and suggested interchangeability, recent studies indicate that significant variations can exist that markedly affect the epigenetic and functional properties of the iPSCs. Through different mechanisms and kinetics, these two reprogramming methods reset genomic methylation, an epigenetic modification of DNA that influences gene expression, leading various groups to hypothesize that the resulting pluripotent stem cells might have different properties. Indeed, a number of reports have indicated that iPSCs often have some DNA methylation patterns which are characteristic of the somatic cell origin prior to induction of pluripotency. This "epigenetic memory" can favor differentiation of the resulting iPSC back along lineages related to the original parent cell and often also results in restriction of alternative cell fates. Indeed, techniques such as high-resolution methylation analyses demonstrated that differences in methylation patterns could consistently distinguish between iPSC and ES cells [17] and produced variety in the level of expression of gene programs. Even subtle changes in expressions levels for just a handful of genes have given rise to the rejection of iPSCs in mouse transplantation models, indicating that some iPSCs are immunogenic and raising concerns about their therapeutic use [18].

In the coming years the efficiency of generating iPSCs and the understanding of the mechanisms of cell programming and reprogramming are likely to improve. However, there are ongoing concerns over safety presenting a significant hurdle before we will see significant progress toward therapies. At this point, considering just the regulatory issues alone, the differences between iPSC and ES cell become the emphasis in calculating the remaining steps necessary to sufficiently characterize the properties and differentiation capabilities of iPSCs as sources of potential commercial therapeutic products. Such studies will be crucial for determining the suitable iPSC types for future stem cell−based therapies for human degenerative diseases.

So far, only a handful of patients have undergone iPSC-based treatments. In 2014 a woman with macular degeneration of the eye received a transplant of iPSC-based retinal cells derived from her own cells. The woman treated showed no apparent improvement in her vision, but the safety of the iPSC-derived cells seemed to be confirmed. In 2017 five additional patients were treated for the same eye condition with iPSC-derived retinal

cells, which were derived from allogeneic iPSC lines (i.e., derived from different donors). According to an article in the January 17, 2018 edition of the Japan Times, however, one of those patients developed a "serious" but non−life threatening reaction to the retinal cell transplant, forcing doctors to remove the transplanted sheet of cells.

Direct reprogramming of differentiated cells

In the last few years a number of studies have shown that a direct route can be taken to convert one differentiated cell to another, without going through an embryonic-like undifferentiated state. Direct reprogramming of differentiated cells has already been demonstrated, as explained earlier and the ability of certain genetic factors to dominantly specify cell fate has been known for some years. However, based on recent advances, this technology is likely to progress significantly over the next 5 years. Direct reprogramming has a number of major advantages, including the potential to produce therapies based on small molecules and/or biologics for in vivo reprogramming. This method would also produce cell therapies without the need to use a pluripotent cell stage; thus greatly reducing the risk of rogue cells leading to uncontrolled cell growth or inappropriate differentiation into an unwanted cell type.

Small molecule-induced differentiation

Small molecules can also induce differentiation and have advantages in terms of the ability of the clinician to control dosage. For example, small molecules have been used to generate iPSCs by acting as substitutes for genetic reprogramming factors. Such approaches offer the longer term potential to activate dormant stem cells in the adult body, and proof-of-concept for this has been most recently demonstrated through the use of a small naturally occurring molecule, thymosin-β4, to stimulate cell-mediated repair of a damaged mouse heart [19]. Other small groups also are making inroads with chemically induced pluripotency, for instance, showing that specific DNA-binding hairpin pyrrole-imidazole polyamides could be conjugated with chromatin-modifying histone deacetylase inhibitors such as Suberoylanilide Hydroxamic Acid (SAHA) to epigenetically activate certain pluripotent genes in mouse fibroblasts [20].

Reimbursement

At the end of the day, life science companies developing new drugs and treatments, including those in the regenerative medicine space, must justify their existence to the shareholders who invested and therefore funded the translation of a therapy from animal models to an authorized therapeutic product. That justification is largely based on the ability of the developing company to sell the product into the marketplace. Accordingly, before deciding to invest in developing a new regenerative medicine product, especially a cellular or tissue product, the company needs to carefully consider the sunk cost to get to an approved product and the resulting cost-of-goods once approved and weigh that against the likelihood of private insurance companies (private payers) and governmental payers (such as Medicare) being willing to reimburse the company for treatment of patients with the approved product, and if so, at what amount. Reimbursement is a multipronged process that requires evaluation of several components: coding, coverage, and payment.

It is the responsibility of companies to demonstrate clinical and economic value for new regenerative medicine therapies. The field of establishing cost−benefit trade-offs associated with new treatments is known as pharmacoeconomics. A pharmacoeconomic study evaluates the cost (expressed in monetary terms) and effects (expressed in terms of monetary value, efficacy, or enhanced quality of life) of a pharmaceutical product. There are several types of pharmacoeconomic evaluation: cost-minimization analysis, cost−benefit analysis, cost-effectiveness analysis, and cost−utility analysis. Pharmacoeconomic studies serve to guide optimal health-care resource allocation, in a standardized and scientifically grounded manner. In this regard, a pharmacoeconomic analysis will determine whether regenerative medicine approaches are clinically And economically effective, capturing such factors as the downstream costs associated with managing disease, assessing direct, and in some cases indirect, costs-associated morbidity and mortality in the patient population. This analysis includes assessing the downstream impact of a new treatment on patients' quality of life and comparing it with the incremental cost of treatment over time. For example, living with reduced vision due to ischemic retinopathy or an amputated limb due to a diabetic ulcer would each significantly reduce the quality of life value for a diabetic patient. When appropriate data are available, it is possible to develop a reliable set of outcomes and economic scenarios, consider trade-offs, and make better decisions regarding the value of a novel therapy.

Emphasis on economic justification for patient care is evident across all aspects of healthcare, from reimbursement decisions by government and private insurance entities to payers demanding data that support treatment pathways, to large payer systems that collect and analyze their own data. Companies are incorporating pharmacoeconomic assessment tools and decision-analysis processes earlier in product development. More frequently, pivotal clinical studies are designed to capture cost data as well as clinical outcomes. Every advanced cellular therapy

developed today will undergo some level of pharmacoeconomic analysis.

Obtaining a medical procedure code is important for reimbursement purposes, and this process begins prior to FDA approval. The Healthcare Common Procedure Coding System (HCPCS), developed and maintained by the Centers for Medicare and Medicaid Services (CMS), is the major medical procedure code system used for billing in the United States. Medicare is the largest single-payer of health-care services in the United States, with almost 50 million beneficiaries and a budget in excess of $425 billion in FY2012. While Congress has the authority to change Medicare benefit categories, CMS makes the decision whether to approve new treatments and these decisions also influence private health plans' coverage decisions, because of Medicare's size. CMS decisions are based on the statutory requirement to cover treatments that are "reasonable and necessary" from a clinical perspective; neither comparative effectiveness nor cost-effectiveness is currently an explicit criterion.

Medical providers submit claims to public and private insurance payers to receive payment for their services. This information is conveyed to payers through codes—numeric and alphanumeric characters which represent a specific service, procedure, or product provided to a patient. Two code sets are used. Current Procedural Terminology (CPT) codes are maintained by the American Medical Association (AMA) and are used to report office visits, surgeries, and other services reported by physicians. Changes to CPT codes (additions, revisions, or deletions) are made annually by the AMA. HCPCS Level II codes are alphanumeric codes maintained by CMS. These codes are typically used to identify supplies, products, and services not included in CPT, such as drugs and durable medical equipment. Changes to HCPCS codes are made quarterly by CMS.

To assess a new technology's clinical effectiveness and safety, CMS and private payers undertake technology assessments. For example, the assessment may determine if a technology achieves particular goals, which may include the following:

1. The technology must have received final approval from the appropriate governmental regulatory bodies, such as FDA.
2. The scientific evidence must allow conclusions to be drawn concerning the technology's effect on health outcomes.
3. The technology's beneficial effects must outweigh any harmful effects.
4. The technology must be as beneficial as any established alternatives.
5. The health improvement must be attainable outside investigational settings.

That being said, even if a product has a code, it is not always reimbursed. Some insurance payers, including Medicare, have separate coverage determinations that indicate when a service is or is not covered. Coverage determinations describe whether specific services, treatment procedures, and technologies can be reimbursed and under what conditions. Most frequently, Medicare coverage determinations are made by local insurance contractors through local coverage determinations and address coverage, coding, and billing guidance. Medicare also issues national coverage determinations for an item or service to be applied on a national basis.

For Medicare, services and products are reimbursed through payment systems determined by the site-of-service. The following payment systems are relevant to regenerative medicine: outpatient prospective payment system for outpatient hospital, ambulatory surgery center services; physician fee schedule for physician office services; and the inpatient payment prospective system for hospital inpatient services. Each payment system is unique and reimbursement strategies will need to be developed with site-of-service in mind.

Often insurers will require companies to demonstrate the cost-effectiveness of their products, especially relative to the costs of existing treatments for the underlying condition. In addition, payers are becoming more interested in the comparative clinical effectiveness of new products versus existing treatments. The burden is on the companies bringing forward new and innovative products to demonstrate why insurers should be paying for them. Accordingly, early in their clinical programs, companies should begin to plan their reimbursement strategy by conducting an initial reimbursement analysis of their product. The following questions will help companies determine where they should focus their efforts.

- *Where will the product be administered?* The reimbursement strategy will vary based on the site-of-service. If the product is performed in different settings (physician's office, hospital outpatient, etc.), it may require several different strategies based on the location where the service is performed.
- *Who is the customer?* Strategies will be different for physicians, hospitals, or specialty pharmacies as each one is reimbursed through different payment systems.
- *What patient population will receive the product?* For example, if the product is intended primarily for an over age 65 population, the payers of concern might be private commercial insurance but more likely would be Medicare and Medicaid.
- *Is the product unique or are other similar products on the market?* If similar products are on the market, it is possible that payment and codes already exist for the product.

- *Does a code exist which accurately reflects the product?* If no code exists, begin the process to secure a new code assignment. If it does exist, is the payment level appropriate for the product?
- *What clinical evidence exists?* Medicare and private payers examine clinical evidence when determining coverage. Companies should begin early with compiling clinical evidence as this will be needed for coverage and coding requests. In addition, the company should compile economic data related to the use of its product.

Emphasis on economic justification for patient care is evident across all aspects of healthcare, from reimbursement decisions by CMS to payers demanding data that support treatment pathways and to large payer systems that collect and analyze their own data. Companies are incorporating pharmacoeconomic assessment tools and decision-analysis processes earlier in product development. More frequently, pivotal clinical studies are designed to capture cost data as well as clinical outcomes. Every advanced cellular therapy developed today will undergo some level of pharmacoeconomic analysis. The following is a checklist for companies, along with timeframes, to help guide executives through the reimbursement process.

- *Conduct a reimbursement analysis and formulate a strategy.* The analysis ideally should be prepared as the company prepares for Phase II clinical trials, but if not, it should be done prior to commencing Phase III trials. The analysis should include an assessment of where and how the product will be administered, how the product will be coded, and how similar/comparative products and existing treatments are reimbursed. Based on this assessment, the company should begin formulating a reimbursement strategy.
- *Gather health economics and comparative clinical effectiveness data.* During Phase II and Phase III trials the company should collect pharmacoeconomic data to demonstrate the cost-effectiveness and clinical effectiveness of the product relative to existing treatments. More and more frequently, payers are expecting companies to demonstrate the "value" of their product prior to reimbursing it.
- *Secure a meeting with CMS (Medicare), private insurers, or both.* This will accomplish two goals. First, it will educate payers about the technology and its clinical and economic values. This is especially important for new technologies such as in regenerative medicine. Second, it will give the company an opportunity to ask questions and get a better understanding of the data needs that payers have when making coverage and payment decisions. This information can be used when designing your Phase II and III trials.

Conclusion

Regenerative medicine is not just a future hope, it is a reality today. Cell-based therapies and products are on the market now and many more are in advanced stages of being tested in patients. These products provide insight into what the future holds in terms of patient health and economic impact. Regenerative medicine is a multidisciplinary field, and to increase the likelihood of success, it is incumbent on those involved to draw on the expertise of a wide range of fields and stakeholders. Collaboration between academia, industry, and clinicians is a vital component for the future success of the regenerative medicine field. This rapidly emerging field of medicine, particularly cell-based therapies, has the potential to deliver dramatic clinical benefits and address important unmet medical needs. It is possible to translate clinical improvement into a robust assessment of the economic benefit derived from superior clinical outcomes.

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Chapter 86

Ethical issues

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Introduction

Case study: The small black box holds a perfectly shaped ear. The scientist at the front of the room explains how it was made. A scaffold of nanoparticles supports fibroblast cells that grew over the form, and the ear now looks and feels actual: it can be transplanted to tissue. For burn patients, this represents an enormous chance and change. It is the prototype of a new genre of medicine, one that uses powerful technologies and methods of bioengineering and cellular biology to transform the matter of the world.

The ear in the box is not a freak example of a new technique. It is, in fact, one of a number of new devices that utilize the convergent technologies of several different fields of science and engineering to create tissue that can mimic the structure and function of the natural world. Other examples include the creation of skin grafts, corneas, bone, cartilage, and, in some pilot studies, bladders. Based on the new technologies of genomics, informatics, nanoscale engineering, molecular biology, and stem cell research, tissue engineering can be said to alter the concept of medicine itself. Instead of treating ailing tissues or organs with drugs intended to repair their structure or function, tissue engineering aims at replacing the diseased or injured or aging tissues of the body with new ones entirely, made from component parts of the material of the world, both naturally occurring and synthetic.

Such an advance heralds a radical new potential ability to heal, a long-awaited solution to several intractable problems, and a serious alternative to cadaveric or living-donor whole organ transplants, which have long been an ethically challenged sector of medicine [1,2]. Yet such a remarkable reconstruction of the human body asks a great deal of any social world into which it is introduced, for it is the body that is the place of the self, the location of the acts of the sacred, and the sensory arbiter of the real. In fact, tissue engineering queries two of the very aspects of our humanity that we consider distinctive: our integral embodiment and our finitude. If we are indeed a collection of replaceable and adaptable parts, some people reason, what is it that separates us from any other engineered machine? If we can engineer, for example, a synthetic and improved lymphatic system, might we improve our chances to adapt to and overcome infectious disease? What other capacities for healing or alteration of our bodies might be prudent? How do we ensure that such changes are indeed ethical?

It is this query that has greeted the new biotechnologies of the body, one based, this chapter argues, in social reactions largely shaped by culture both ancient and contemporary. We then ask the following: What are the ethical challenges to the field of tissue engineering? Does tissue engineering raise new ethical issues, or is it a description of one of the modalities enabled by the convergence of other technologies that have been understood to be individually ethically freighted?

In this chapter, I suggest using an established ethical framework that was suggested in 1999 by a committee of the American Association for the Advancement of Science on inheritable genetic germline modification and used far more widely by the field of bioethics to assess new technologies. We then review the responses given to new technologies in the past from a variety of sources in bioethics, philosophy, and theology. Finally, we reflect on how the legal and regulatory structure for tissue engineering impacts on our reflections on ethical norms [3].

Research evaluation (adapted from the AAAS Working Group on Human Inheritable Genetic Modifications 1998–2000 [3])

- Are there reasons, in principle, why performing the basic research should be impermissible?
- What contextual factors should be taken into account, and do any of these prevent the development and use of the technology?

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- What purposes, techniques, or applications would be permissible and under what circumstances?
- On what procedures and structures, involving what policies, should decisions on appropriate techniques and uses be based?

Are there reasons, in principle, why performing the basic research should be impermissible?

Principled reasons for objections to basic research are extremely difficult to conceive in research that is, by its very nature, intended to be translational and clinical. Yet ethical objections to the manipulation, replacement, and engineering of human tissue can be seen as part of a long continuum of dissent about medical technology that began to assume full voice in the 1970s, when successful genetic manipulation of bacterial genomes became possible [4].

All new technology raise new challenges—in particular, technology that refashions the embodied self, become a part of the "self" and the identity of the subject, and seem to raise the deepest anxieties. Even tissue engineering, an emerging field with clear targets, clinical successes, and patient needs, will raise familiar concerns.

First among these is the argument that humans possess an essential nature and live within an essential natural order that cannot be altered without harm. For Lewis [5], this is expressed as a concern that the very acts of rational science—dissection, analysis, and quantification—are a violation of the sacred integrity that lies behind all of nature:

> *Now I take it that when we understand a thing analytically, and then dominate and use it for our own convenience, we reduce it to the level of 'nature', we suspend our judgments of value about it, ignore its final cause (if any), and treat it in terms of quantity. This repression of elements in what would otherwise be our total reaction to it is sometimes very noticeable and even painful: Something has to be overcome before we can cut up a dead man or a live animal in a dissecting room.*

For Lewis the understanding of the body as replaceable is disturbing:

> *The real objection', he says, 'is that if man chooses to treat himself as raw material, raw material he will be, not raw material to be manipulated by himself as he fondly imagined, but by mere appetite' (*p. 274*).*

What Lewis imagines is that new transformative technology will be manipulated by "controllers" who will eventually transform man into mere matter, and, as a scholar more interested in metaphysical transformation than corporeal change, this is disturbing.

Bioethicist Callahan [6] echoes Lewis' concern, agreeing both with the argument that limits need to be placed on what is "decent" or "proper" to do to nature, and in the sense that such action is a part of a larger danger. Both are worried that power in the hands of scientists represents not only an altruistic desire to heal suffering but also a deeper desire for power. When such power is left to scientists, they argue, scientist may be driven by the interests of the elite, who increasingly fund their research projects, or even by a State, that may well (and historically has) use science to manipulate and control populations. Callahan argues

> *The word 'No' perfectly sums up what I mean by a limit – a boundary point beyond which one should not go … There are at least two reasons why a science of technological limits is needed. First, limits need to be set to the boundless hopes and expectations, constantly escalating, which technology has engendered. Advanced technology has promised transcendence of the human condition. That is a false promise, incapable of fulfillment. … Second … limits (are) necessary in order that the social pathologies resulting from technologies can be controlled. … [W]hile it can and does care, save, and free, it can also become the vehicle for the introduction of new repressions in society.*

These objections are not new, nor were they made in response to new advances such as tissue engineering. They were made well over 30 years ago, and yet are still made, despite, one may note with some irony, 30 years of medicine that have indeed seen rapid and successful advances, without their being used by the state for repression and without any fundamental change in the capacities for intellectual and spiritual self-possession.

Nevertheless, despite this empirical reality, powerful arguments of opposition to the manipulation and replacement of tissues and organs continue, with some people continually worried that "perfection" itself or dominance, or privilege is what is sought even when, consistently, healing remains the goal of medical research. Such critics, many from the disability community, raise principled objections to the use of tissue engineering if the goal is to alter the disability. Activists in the deaf community, for example, defend their disability as a culture and a language exchange, not as a loss of function. Others are concerned that our society's focus on "fixing things" will allow a devaluation of the persons that currently bear the broken bodies and parts. Bioethicist and disabled activist Adrienne Asch suggested that there is a "troubling side to every cure, that those of us who are uncured are seen as less valuable, perhaps even expendable."

For bioethicist McKenney [7] a community needs to "embrace brokenness" and to "deny that the worth of one's life is determined to how closely one conforms to societal standards of bodily perfection." McKinney is also concerned that if medicine is successful, it will create a social and economic system that "virtually demands that we be independent of the need to care for others."

Duty and healing: natural makers in a broken world

While the opposition to medical technology has indeed been persistent, it also has not been unchallenged. For many the response lies in the nature of brokenness and the human duty to respond to the need of the suffering other [8,9]. The principle at stake in the assessment of tissue engineering as an ethical act is not how its use might potentially violate an abstract community in the future, but the actual problem of what one must do as a moral being when one's neighbor is in need. In this important sense the duty to heal cannot be overridden by a "sense" of discomfort (as Lewis notes in the earlier quote).

It is the nature, goal, and meaning of science to address the human condition in all its yearning and capacity for defeat and failure. In this sense, there is no principle objection to the science of tissue engineering, and in fact there may well be a strong moral imperative to develop the technology.

Fletcher [10], in speaking of an earlier generation of medical technology and answering the critics of science, wrote

> *'The belief that God is at work directly or indirectly in all natural phenomena is a form of animism or simple pantheism. If we took it really seriously, all science, including medicine, would die away because we would be afraid to 'dissect God' or tamper with His activity'. 'Every widening and deepening of our knowledge of reality and of our control of its forces are the ingredients of both freedom and responsibility'.*

Gerald McKinney and Lawrence Childress use an argument that is rooted in Christian moral theology: that since human persons are fallen creatures in a fallen world, we cannot really be counted on to know the right and the good. God, who is transcendent, from this fallenness, has set us in this place, not essentially to alter it toward our own transonic, but to find its meaning and purpose. Yet the traditions of other faiths differ. For Jewish and Islamic theorists the world is morally neutral. Human persons may—and will—fail in their aspirations but can be trusted to have the capacity for moral behavior and moral yearnings. Finding meaning in suffering is not the core task. The task is to alleviate suffering, which is understood as chaotic, meaningless, and agonistic. Hence, many of the core objects in principle are rooted in religious constructions and understandings.

To make is to know: notes on an old problem about knowledge

The classic debates of the 1970s were largely in response to the concept of the first generation of gene editing and proposals for gene therapy. The issues they raise are not the only a set of problems engendered in the history of ethical responses to the technological gesture at the heart of tissue engineering. At stake as well is the special kind of knowledge that such making implies. For Aristotle and the Hellenists, useful knowledge, "practical wisdom," was phronesis. Phronesis implied actually doing an act; making, in order to know. The act of making, not the act of perception or contemplation alone, was how wisdom and, indeed, rationality and power were achieved. Hence, making new tissue is a somewhat different moral gesture than curing the body by altering it with drugs that essentially allow the body to heal itself.

Second, the use of technology within the body of the patient is a different matter than the use of technology essentially to enhance the body of the practitioner. For all earlier technology the thing that was changed or enhanced was the sense perceptions of the doctor. Stethoscopes and otoscopes allowed the sounds of the body to be more audible. X-rays, CT scans, and MRIs allow the inner vistas of the body to be revealed. EEGs and EKGs allow the electrical currents that animate the central and peripheral nervous systems to be charted in quantifiable units. Microscopes allow invasive bacteria to be seen at the microscopic and, increasingly, molecular levels. These earlier technologies extended the reach of what Bacon increasingly trusted and that the Greeks did not: the perception and observation of the phenomena of the world and the perception of the outcome of its deliberate perturbation.

Bacon's method presupposes a double empirical and rational starting point. True knowledge is acquired if we proceed from lower certainty to higher liberty and from lower liberty to higher certainty. The rule of certainty and liberty in Bacon converges …. For Bacon, making knows and knowing is making (cf. Bacon IV [1901], 109−110). Following the maxim "command nature … by obeying her" (Sessions [11]; cf. Gaukroger [12]), the exclusion of superstition, imposture, error, and confusion are obligatory. Bacon introduces variations into "the maker's knowledge tradition" when the discovery of the forms of a given nature provide him with the task of developing his method for acquiring factual and proven knowledge [11].

Thus the world is known by understanding the parts of the world and, from that, theorizing (knowing), by induction, to principles or axioms or laws of nature, physics, and chemistry. In contemporary science, knowing is done largely by "unmaking," by deconstruction of the component parts in ways scientists of Bacon's era were unable to imagine. Many of these "unmaking" techniques, such as the splicing of alternative DNA and the manipulation of cellular structures, allow a sense of inherent interchangeability, as if the real and the person were merely a set of Lego parts awaiting clever recombination.

What is a thing? The perils of deconstruction

Making actual tissue in mimesis of the real tissue of the actual body extends the Baconian act in radical ways. Here, the experimental perturbation is the unmaking of tissue and the remaking of tissue, only in more controllable form. This cannot help but excite concern about the nearly infinite possibilities for technological shaping of the self. Heidegger asks, "What is a thing?" and in so reflecting understands a thing as an object separate from the self. But what of a made thing, an object that becomes the self?

The technology of the alteration of the patient is distinctive. Devices for altering the functioning of the body that become a part of the body and are actually a tissue of the body are a step beyond the idea of a device held within the body. This is important in any ethical assessment of the technology, because the patient's consent and participation are needed for the final act of the technology to be completed. Such an event only happens in a specific context, for technologies, patients, and practitioners operate in a social, religious, and economic context. Here we turn to the second ethical consideration.

What contextual factors should be taken into account, and do any of these prevent the development and use of the technology?

Tissue engineering is a complex procedure still in experimental stages. Yet to be an ethical technology, it must be directed toward accessibility, just distribution, and efficacy. Hence, the troubling context of widespread healthcare disparity is a problem not only for this advanced technology but for all newly emerging technologies. Emergence into an unjust world asks certain moral questions of new technological advances. First among these is the query about burdensomeness versus benefit in a context in which the vast majority of the world's people suffer from easily treatable infectious diseases such as tuberculosis, malaria, AIDS, and infant diarrhea. How can tissue engineering be justly promoted in the face of other, pressing needs?

This objection can typically be met by noting that it would be deeply inappropriate to withhold medical knowledge until the world is entirely perfected, and that applications not only will be increasingly available to the poor (as in vaccines, once rare) but also that the very process of research has typically uncovered new and useful ways to understand disease.

The goal of tissue engineering is the widespread use of the technique. Unlike solid-organ transplants, which would always require significant resources far outside the capacities of developing-world clinics, tissue replacement, stem cell therapies, and other transportable therapies are designed for widespread use. The possibility to create a method for allografts that uses the patient's own cells and the possibility for autologous cells to provide an "off-the-shelf" source of tissue may provide the basis for access (but only if research priorities are discussed in advance of design), a process, as we describe later, that will need careful support and monitoring. The question of how to achieve this and how to enable a more just use of each technology has not yet been solved.

A second contextual factor for tissue engineering is that all human tissue is marked by its genomic identity. It is the very nature of cells that allows them to copy and reproduce and to carry identifying markers linked to some person somewhere. In the past, such use has raised serious objections. Such tissue can be traced and known, which may have implications for the person who is the source of the tissue, raising significant new issues in genetic privacy for the donor.

Further, whose is the tissue that is derived from the cells of a particular body? Who should have the rights to, and a fair share of, the profits derived from its use? In the seminal case in the field, Moore v. the Regents of the University of California [13], the issue of ownership was addressed. In this case, Mr. Moore had his T cell lymphocytes taken from his spleen during the course of treatment for hairy-cell leukemia, cells that proved effective in deriving resistant cell cultures. Patented after manipulation to make a new "product," the cells were indeed profitable. Mr. Moore's complaint was that he was not informed of, much less a part of, the scientific enterprise and the lucrative payout for his cells. The case was decided in favor of the research labs. But in the ensuing decades, alert patients with unique cell types or unusual cancers who sought for research have been selling their materials as personal possessions to the lab that wishes to procure them. Ownership is limited, however, by the common law of the United States and the European Union which constrains this ability to claim tissue as property. The goals of such restraints were put into place to prohibit the buying and selling of human tissue and organs, for fear, given the desperation of the poor, that selling the bodies of the poor would become permissible and led to their exploitation. Thus the entire process that allows for the derivation of tissue sources needs to be noted. The current context for tissue donation is a mixed system. Organs, tissues such as blood, corneas, and marrow are donated or exchanged without compensation. Gametes, however, are another matter entirely. Because the use of human sperm and eggs emerged in the context of fertility treatment and because this treatment was largely conducted in stand-alone, private clinics that functioned

without public oversight or regulation, the marketplace standards prevailed. What originally began as a compassionate exchange of gametes between family members when an infertile couple could not conceive quickly changed into a robust marketplace in human gametes. As of this writing, international standards prohibit the use of marketplace incentives for gametes or embryos.

A final context for the debate about the ethics of tissue engineering in general is the special case of human stem cells to make tissues. Because some applications of tissue engineering use stem cells as a part of the method of treatment [14], the debate about the ethics of the use of human stem cells is directly adjacent to this technology. For many the origins of tissue matter a great deal. For some Christians, many Roman Catholics, and some Hindu sects, the destruction of the human embryo, even at the blastocyst stage, is tantamount to killing. For these faith traditions the derivation of stem cells from embryos is always impermissible. For many other faith traditions, such as Judaism, Islam, Jainism, Buddhism, Confucian philosophy, and Daoism, the use of these cells is permissible within certain constraints, as we will see later. For all faith traditions, however, the manipulation of adult somatic cells in their precursor form is completely sanctioned. Precursor cells are not as flexible as pluripotent cells, and it is that very pluripotency and immortality that are important in tissue engineering. These factors raise concern. Yet the contextual factors alone do not prohibit entirely the use of this technology, for justice in distribution, the possibility of the loss of genetic privacy, and the controversy over stem cell research when pluripotent embryonic cells are used to affect many aspects of the new techniques in medicine. Hence, we turn to the third major issue.

What purposes, techniques, or applications would be permissible and under what circumstances?

Many of the salient, justifying arguments for the use of tissue engineering hinge on the telos, or goal, of the treatment: if the goal is to cure or treat human disease, the benefits will outweigh the burdens of the work—controversy, cost, and difficulty. Clearly, then, tissue engineering ought not to be used in a trivial or wasteful fashion. Human tissue is understood by many as deserving a special sort of "respect" [15].

This proviso may not be so simple, for a core problem in genetic engineering has been the use of the technique for "enhancement" of human characteristics or traits. The initial ethical discussions about therapeutic uses of medicine versus cosmetic ones imagined ethical bright lines that would define the boundary between the use of such technology to restore "species normal functions" [16] for each tissue and for the person as a whole. Yet medical practice has long gone beyond these lines, using surgery, for example, for cosmetic purposes. Will it be possible to restrict tissue replacement to burn victims, spinal cord injury, and diabetics? How can such a distinction be made?

Some tissue-replacement therapies, such as the use of skin grafts for full-face transplant, may also raise questions about the nature of identity. Indeed, the notion of a full-face transplant alerted us to the depth of resistance to identity—altering tissue replacements. (Could persons use any face? What if persons in need of facial transplants wished to change ethnicity? Should faces "match," and why?) Like many other aspects of this technology, this tension about identity was not new, only heightened. For example, the first years of organ transplant raised the same issues for recipients of hearts, a key aspect of identity in many cultures. If the face is our key determinant of the self in modernity and, even more so, if the brain is such, then how are we to understand the use of tissue engineering to transform identity?

Hence, linked inexorably to this technology are larger considerations of the use of tissue engineering for neuroscience, both for therapy and for enhancement. The applications of tissue transplant in Parkinson's disease are important. Yet will there be concern about this use of the neurons of a stranger in the brain of the self? Of all the possible uses of tissue engineering, the ones that may alter consciousness and memory are the most troubling. (What capacities or memories could neurons store?) Here, the need for restrictions on applications may be the clearest, yet it is not clear who ought to decide and who ought to ensure that the restrictions on unethical applications are maintained. By on what criteria will such limits be set?

New research possibilities also offer applications to engineer gametes for use and storage. Engineered follicles may now be saved, frozen, matured, and used in animal models to create the possibility of human fertility after cancer chemotherapy or other environmental risk.

With this, as with all such technology, there will have to be careful attention to how the market may drive technology toward specific research goals rather than others or to whether research goals will be framed only by the values of profit and efficacy and not by ones of more general interest: compassion, healing, and solidarity. The powerful applications and the potential for widespread use itself create the possibility for serious conflicts of interest, for serious market forces may be the core drivers of technology, especially in an aging population with increasing needs for all manner of new tissues and organs. This turns us to the consideration of our final set of issues.

On what procedures and structures, involving what policies, should decisions on appropriate techniques and uses be based?

Much of the first reviews of the ethical issues in tissue engineering have in fact focused on the issues of policy: safety, patents, and gating. Products and drugs are typically controlled via four levels of restraints. The first is elaborate premarket gating, first involving animal models and then typically done for pharmaceuticals in a decade-long series of tests, phased to test the drug on an increasing but controllable number of human subjects. Such trials must be gender balanced, and subjects must give full, informed consent and be able to leave the trial at any time (which could be difficult in the cases of implanted tissues).

The next gating is the system of intellectual property. Patents and licensing control the use of the products, and even the replication of the experiments. The next gating is that of financial backing. To perform the enormous clinical trials, to do premarket investigation, and, of course, actually to make and sell the product require a production apparatus, which must be assembled and supported. Finally, each drug or device must be approved for use by the insurers. As Smith (2004) notes, tissue engineering faces a gauntlet of issues and a "new order of magnitude in interactions and science patents." In addition, notes Smith, the "things" engineered are hybrids of two jurisdictions, that of drugs and that of devices.

Are genetically engineered insulin cells a drug, like insulin, a device, like a stent, or a biologic? Unlike stents, which are entirely synthetic, tissue engineering uses actual human cells, only manipulated in de novo ways.

Standards will need to be set for safety, efficacy, and fair use. Standards for clinical use, for clinical trial, and tissue stability and purity will be needed for the research and application to be safe.

Getting informed consent in this case will present significant challenges. Patients in need of organs, for example, are particularly desperate, and their consent may be deeply affected by their utter lack of options. Eight percent of the medical system is already devoted to organ transplantation, and the lack of organs is an overwhelming problem for nearly half of the patients hoping for transplants [17]. Yet the first year of the use of engineered tissue will be experimental and will need to be conducted under the strongest possible set of NIH guidelines. How the first trials of engineered tissue are conducted will set the tone and the future for all subsequent use.

The question of policy and the regulation of policy are manifested in many of the first documents that evaluate the ethical and legal implications of tissue engineering. While, as Smith notes, the United States faces a complex regulatory system, the European Union has regulated such research products as medical products, and these will fall under the regulatory gaze of the European Medical Evaluation Agency. In both the United States and the EU, the synthetic nature of tissue engineering, the very de novo quality of the work, and the uneasy greeting that met genetically modified food have created serious political opposition. Policies need to be crafted with transparency and full public participation, for such research needs not only public funding but public understanding of complex theory and practice of tissue engineering—what promises it can hold and what cautions need to be applied prior to use. Policymakers will need to attend to calls for justice in distribution, as was noted earlier, and will need to set in place structures for regulation.

How can new technologies best be regulated? I contend that a full array of regulatory structures can be employed. First among these are local committees, IRBS, and local review boards. The National Academies have played a large role in policy writing for both recombinant DNA and for stem cell research and, in both instances, called for special, national, ongoing oversight on such research. It would be prudent to reflect on the need for such a process for tissue engineering, for established structures largely address issues involving the use of donated tissue, not engineered tissue. Structures that protect human subjects also need strong enforcement, as noted earlier, both for donors and for recipients of tissues.

But regulation, government oversight, and market forces can only go so far in shaping just research goals and commitments. The goal of ethics is to develop moral agents who are aware of a constancy of duty toward subject and to humanity, who not only follow rules correctly but also, given the chance and grace to work at the frontiers of science, act with courage and decency in their research.

Conclusion

Tissue engineering suggests that an old dream—the replacement of human body parts—may be realized. While any sober and reflective scientist understands the long way to success of this idea, the science described in this volume clearly suggests that our society is on the road to the enactment of the possibility.

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Index

Note: Page numbers followed by "*f*" and "*t*" refer to figures and tables, respectively.

A

ABICUS, 930–931
Absorbable collagen sponge (ACS), 1209, 1211–1212, 1228, 1512–1513
Absorptive cells, 709–710
AC. *See* Articular cartilage
Accelerated approval, 1564*t*, 1565, 1565*t*
Accordion-like honeycomb pores, 165
Acellular biologic scaffolds, 833
Acellular composite skin graft, 1321
Acellular iNP matrix, 1067*f*
Acellular, scaffold-based products, 1503
Acellular smart matrix tissue-engineering approach, 1322
Acellular synthetic polymeric brain implants, 1030
Acellular tissue engineering, 864, 1324, 1331
Acellular vagina matrix (AVM), 867
ACE-Mg, 1096–1098
Acetaminophen (APAP), 1447, 1449, 1452–1453
Acetate-modified hyaluronic acid, 1059
Acetylcholine, 220–221
Acetyl-*l* carnitine, 1096–1097
Achilles tendon, 899–900, 990
ACI. *See* Autologous chondrocyte implantation
Acoustic trauma, prevention of, 1096–1097
Acquired loss of auditory nerve connections to hair cells, 1098–1099
Acquired sensory hair cell loss, protection from, 1093–1094
Acral skin, 1296
Actin, 219, 239
Activated calpain, 1095
Activated platelets, 1310
Activator-inhibitor model, 44
Active EGF-like fragments, 1262
Active tactile sensing mechanism, 1323–1324
Activin A, 836*t*
Actomyosin, 218, 247–248
Actomyosin-dependent physical distortion, 229
Acute and chronic pulmonary disorders, 1253
Acute kidney injury (AKI), 825, 829
Acute liver failure (ALF), 737
Acute lung diseases, 1262
Acute lymphoblastic leukemia (ALL), 770–771, 873
Acute/repetitive blunt joint trauma, 968–971
Acute rejection, 1494
Acute wound healing, 1309, 1333
Acute wounds, 1317–1318
AD. *See* Alzheimer's disease
ADAMTS5, 955
Adaptation state, vision enhancement systems, 1164
Ad capsid, 497
Additive manufacturing (AM) techniques, 924–925
Adeno-associated virus (AAV), 493, 499–500, 526–528, 954, 1099, 1156–1157
  capsids, 529
  targeting of, 505
  vectors, 499–500, 538
Adeno-associated virus type 2 (AAV2), 1175–1177
Adenocarcinoma, 847–848
Adenosine triphosphate (ATP), 68
Adenoviral-mediated gene transfer, 954
Adenoviral pseudotyping, 529
Adenoviral vectors, 527–529, 954
Adenoviruses, 497–499, 527–528, 1210–1211, 1232
Adequate oxygen perfusion, 1380
Ad gene transfer vectors, 497
Ad5 genome, 497
Adherens junctions, tension sensing at, 241*f*
Adherent culture technique, 1121
Adhesions
  cell–cell, 240
  cell–substrate, 240–241
  focal, 240
Adhesive glycoproteins, 123–127
Ad-IL-1 receptor antagonist (Ad/IL-1Ra), 954
Adipocytes, 559, 1406–1407
Adipogenesis, 1406–1407
Adipose-derived mesenchymal stem cells, 886, 899–900, 948, 999, 1138–1139, 1155–1156, 1322
Adipose-derived stem cell (ADSC), 162, 876–877, 919, 1225, 1227*t*, 1360, 1376–1377, 1395, 1424–1425
Adipose-derived stromal cells (ASCs), 1010–1011, 1280, 1322, 1357*f*
Adipose tissue, 564, 1409
A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4), 948–952
ADM3100, 837
Adnexal structures, 1487
Adoptive T cell therapy (ACT), 770–771
Adriamycin, extravasation of, 1343*f*
Adsorbed proteins, synthetic polymers with, 282–283
Ad tropism, 502
Adult and fetal stem cells, 826
  amniotic fluid stem cells, 829
  mesenchymal stem cells, 829
Adult bone marrow (BM), 917–918
Adult-derived induced pluripotent stem cells, 1012
Adult fibroblasts, 1319–1320
Adult hemoglobin (HbA), 766–767
Adult mesenchymal stem cells, 1501
Adult progenitors, 744
Adult respiratory distress syndrome (ARDS), 1260, 1265
Adult stem cells (aSCs), 7–8, 23, 849, 1289, 1537–1538
Adult tissue-resident progenitor cells, 1011
Adult vascular endothelial cells (VESCs), 1263
Advanced BioHealing, 1337
Advanced Cell Technology, 1578–1579
Advanced therapy medicinal product (ATMP), 1504, 1506
Advanced Tissue-engineered Human Ectypal Network Analyzer (ATHENA) program, 1454
Ad vectors, 497–498
  nonhuman primate-derived, 498–499
  targeting of, 502–505
Adventitia, 617
Adverse events (AEs), 379, 1556–1557, 1567
AFSCs. *See* Amniotic fluid stem cells
Agarose, 264, 895–896, 941*t*, 984*t*, 1065–1066, 1068*f*, 1502
Agenesis, laryngotracheal, 462–463
Age-related hearing loss (ARHL), 1093
Age-related macular degeneration (AMD), 423–424, 1121, 1145, 1147, 1148*t*, 1149*f*, 1156–1157, 1163–1164, 1167, 1172–1173
  as cellular therapy for, 1147–1149
  prevalence, 1147
  severity, 1147
  wet/exudative form, 1147
Aggrecanase, 948–952
Aging, 889, 1576*f*

Aging (*Continued*)
epithelial stem cells in, 1298–1299
population, 1573–1575, 1576*f*
Aging-related cell density losses, 937
Air–liquid interface (ALI), 1119, 1538–1539
Airway reconstruction, 462–463
Alanine aminotransferase (ALT), 379
Aldehyde dehydrogenase (ALDH) activity, 828–829
Alexander's disease, 436
ALF. *See* Acute liver failure
Alginate, 324, 562–563, 669, 866, 929, 941*t*, 984–985, 984*t*, 1053*t*, 1066–1067, 1068*f*, 1208–1209, 1241, 1399, 1403, 1404*t*, 1423*t*, 1502
Alginate-based bioink with human endothelial cells (ECs), 1395
Alginate-encapsulated hepatocytes, 1403
Alginate hydrogels, 1195–1196, 1234, 1403–1406
Aliphatic polycarbonates, 330, 330*f*
Aliphatic polyesters, 326–330, 561
Alkaline phosphatase (ALP), 888
Alkaline phosphatase-expressing cells, 712
Allo-antibodies (Abs), 374
AlloDerm, 1214–1215
Allogeneic cells, 405, 889
source, 1488
transplants, 1577–1578
Allogeneic granulocyte transplantation (Gtx), 778
Allogeneic human amnion epithelial cells, 458
Allogeneic keratinocytes, 1485
Allogeneic limbal stem epithelial cells, 1137
Allogeneic mesenchymal stem cells, 891
Allogeneic tissue engineering, 1488
Allogeneic workflows, 1470, 1471*t*
Allogenic bone grafting, 926–927
Allogenic cell transplantation, 1236–1237
Allogenic uterus transplantation, 863
Allografts, skeletal tissue engineering, 1007
Alopecia, stem cells in, 1299–1300
Alopecia areata, 1300
α-actin, 875–876
Alpha 2 adrenergic agonists, 1153
Alpha AMS, 1169
α2β1collagen receptor, 1311, 1314–1315, 1317
α2β1 integrin, 1484–1485
α2β1 integrin receptors, 1317
α-Catenin, 240
Alpha fetal protein (*AFP*), 1430–1431
Alpha-galactosylceramide (α-GalCer), 775–776
α-Minimum Essential Medium (αMEM), 885*t*, 886
α-Smooth muscle actin (α-SMA) expression, 219
ALS. *See* Amyotrophic lateral sclerosis
Aluminum nitride (AlN)
microelectromechanical systems xylophone, 1101–1102
Alveolar bone, 1223–1224
Alveolar capillaries, embryonic development of, 1258–1259, 1259*f*
Alveolar clefts, 1203, 1205*f*
Alveolar epithelial progenitor (AEP) cells, 1261
Alveolar epithelium, lung bud to, 1256*f*
Alveolar type I (AT1) epithelial cells, 1256–1257, 1259, 1261, 1276–1277
Alveolar type II (ATII) epithelial cells, 1256, 1259, 1261, 1276–1277
Alveologenesis, 1259
Alzheimer's disease (AD), 436, 777–778, 1025
Ambivalence, 1364
AMD. *See* Age-related macular degeneration (AMD)
AmEc for subnormothermic perfusion of lung, 794–795
Ameloblast, 1190
Ameloblastoma of mandible, resection of, 1202*f*
Ameloblasts, 1187–1189
Amikacin, 1094–1095
Amines, 532–533
Amino acids, 318, 534–535, 1462
Aminoglycosides, 1094–1095
Amniotic fluid (AF), 458, 848
Amniotic fluid stem cells (AFSCs), 23, 458–459, 462, 826, 829, 876–877
Amniotic membrane, 458, 1118–1119, 1149
Amyotrophic lateral sclerosis (ALS), 448–449
Analog integrated circuit model, 1102
Anastomosis, 16
Anchorage-dependent cells, 745
Ancillary materials, 1470–1472
Androgenetic alopecia (AGA), 1299–1300
Androgen-replacement therapy, 873–874
Aneurysmal dilatation, bioresorbable grafts, 625
Angiochip, 608
Angiogenesis, 16, 102, 109–110, 288–289, 1014, 1124, 1166, 1230, 1258, 1262, 1280, 1312–1313, 1315–1316
induction of, 265–267
therapeutic, 266
Angiogenic medium, 1280
Angiogenin, 563
Angiopoietin, 1315–1316
Angiopoetin-1 (Ang-1), 1262
Angiopoietin 1-tie 2, 1280
Angiotensin II, 836*t*
Animal cell, generalized, 66*f*
Animal-derived serum, 887
Animal-free food, 1370
Animal models, 435, 565–566, 1444
intervertebral disk (IVD) regeneration, 955–956, 956*f*
for lower urinary tract reconstruction, 853*t*
neural regeneration in, 480
oral and maxillofacial surgery, 1215–1216, 1215*f*, 1216*f*
spinal cord injury, 1052–1054, 1054*t*
in utero transplantation experiments in large preclinical, 372–373
Ankylosis, 1228
ANNs. *See* Artificial neural networks
Annulus fibrosus (AF), 938–940, 938*f*, 942–944
repair and regeneration, 942–944, 942*f*, 943*f*
Anomalies, congenital, 457
Anterior cruciate ligament (ACL), 995, 997
injury, 968
reconstruction, 995, 998–999
Anterior lumbar spinal fusion, 927
Anthropogenic methane, 1369
Antibody-dependent cellular cytotoxicity (ADCC), 773–775
Antibody manufacturing, 1470
Anti-CD19 CAR T cells, 770–771
Anticoagulation, 638–639, 667–668
Antigen-presenting cells (APCs), 404, 773, 1338
Antigens, donor, 405
Anti-HE4 antibody, 836*t*
Anti-IL-6 therapy, 770–771
Antiinflammatory agents, 967, 974, 1093, 1097
Antimicrobial peptides and histones, 1191–1192
Antiototoxic therapy, 1096
Antioxidants, 967, 972, 1095–1097, 1150
Antiplatelet agents, 627
Aortic endothelial cells, 205–206
Aortic heart valve, 635
Aortic stenosis, 637
Aortic valves, bicuspid, 637
APCs. *See* Antigen presenting cells
Apligraf, 1337, 1339–1347, 1340*f*, 1347*f*, 1483, 1489, 1493
diabetic neuropathic foot ulcer, 1340*f*
histological appearance of, 1335*f*
Apoptosis, 100, 104, 829, 1095, 1097, 1315, 1452
cellular, 167
APQ. *See* Aseptic process qualification
Apraclonidine, 1153
Aqueous polymer, 1054–1055
Arenicola marina erythrocruorin (AmEc), 793
Arginine–glycine–aspartic acid (RGD), 896, 899–900, 1015, 1235–1236, 1323, 1334, 1398
Argus 2, 1168
Argus II device, 1178
Argus II retinal implant, 1170
Armed Forces Institute of Regenerative Medicine (AFIRM) grants, 1473–1474, 1477–1478
Armored chimeric antigen receptors, 771–772
Arterial myofibroblasts, 643
Arterial plaque, 1434–1435
Arterial thrombosis, 1434–1435
Arteriogenesis, 621
Arthrodesis, 929–930
Arthroplasty, 930
Arthroscopic drilling, 973
Articular cartilage (AC), 157, 163–164, 1406–1407, 1407*f*
Articular cartilage injury, 967–968
antiinflammatories, 974
artificial matrices, 973

cell transplantation, 973
chondral injuries, 971
growth factors, 973–974
and joint degeneration, 968
matrix and cell injuries, 970–971
mechanisms of, 968–970
osteochondral injuries, 971–972
periosteal and perichondrial grafts, 973
preventing joint degeneration, 972
promoting articular surface repair, 972
response of, 970
subchondral bone, penetration of, 972–973
Articular cartilage products, tissue engineering of, 1204, 1499
autologous chondrocyte implantation products, 1503–1507
BioSeed C, 1504
Cartipatch, 1505
CARTISTEM, 1505
ChondroCelect, 1504
Chondron, 1505
clinical outcomes, clinical factors affecting, 1508–1509
contraindications, 1505–1506
indications for, 1505
long-term results of, 1508
MACI, 1503–1504
noninferiority studies, 1506–1507
Novocart 3D, 1504
Novocart Inject, 1504
randomized clinical trials, data from, 1507–1508
Spherox, 1504
surgical steps, 1506, 1506*f*, 1507*f*
bioreactors for, 1502–1503
cartilage defects
pathophysiology, 1499–1500
surgical treatment options for, 1500
cells for, 1500–1501
for orthopedic reconstruction, 1500
scaffolds for, 1501–1502, 1501*t*
acellular, scaffold-based products, 1503
agarose and alginate, 1502
autologous chondrocyte implantation, clinical generations of, 1503
collagen scaffolds, 1501–1502
hyaluronan, 1502
particulated autologous/allogenic articular cartilage, 1503
scaffold-free three-dimensional systems, 1502
synthetic polymers, 1502
Artificial capillaries, 1374–1375
Artificial cochleae, 1093
Artificial corneas, 1135
Artificial extracellular matrices, biomaterial scaffolds as, 139–140
Artificial intelligence (AI), 1472–1473
Artificial liver (AL), 738
Artificial neural networks (ANNs), 46
Artificial neurites, 1174
Artificial primordia, 1189
Artificial thymic organoid (ATO), 684–685
Aryloxyphosphazenes, polymers of, 332
Ascorbate, 888
ASCs. *See* Adipose-derived stem cells
Aseptic process qualification (APQ), 56–57
Aseptic techniques, 1562
Asherman's Syndrome, 863
Asia Pacific Economic Cooperation (APEC), 1568
Aspirin, 1095–1096
Assistive technologies (AT) software, 1040–1041
Astemizole, 1454
Astrocytes, 1050–1051
induction of reactive, 269
ATDC5 cells, murine chondrocytic, 43*f*
ATO. *See* Artificial thymic organoid
Atoh1, 1100
Atorvastatin, 1449
ATP, 1310
Atraumatic osteonecrosis of femoral head, 930
Atresia, esophageal, 461–462
Atrioventricular (AV) heart valves, 635
Atrophic nonunion, 929–930
Atrophy and exercise, 1380
Auditory nerve (AN), 1093
replacement, 1101
Augment endogenous bone regeneration, 1512–1513
AutoCAD, 295
Autogenous AV fistulas, 620
Autogenous cellular population, of TEBV, 624*f*
Autografts, skeletal tissue engineering, 1007
Autologous bone grafting, 926–927
Autologous bone marrow–derived mesenchymal stem cell, 994–995, 1347–1348
Autologous chondrocyte implantation (ACI), 892–893, 930–931, 979, 985, 1499, 1503–1507
BioSeed C, 1504
Cartipatch, 1505
CARTISTEM, 1505
ChondroCelect, 1504
Chondron, 1505
clinical application of
contraindications, 1505–1506
indications for, 1505
surgical steps, 1506, 1506*f*, 1507*f*
clinical generations of, 1503
clinical outcomes, clinical factors affecting, 1508–1509
first-generation, 1503, 1507–1508
long-term results of, 1508
MACI, 1503–1504
noninferiority studies, 1506–1507
Novocart 3D, 1504
Novocart Inject, 1504
randomized clinical trials, data from, 1507–1508
second-generation, 1503, 1508
Spherox, 1504
third-generation, 1503–1504
Autologous chondrocytes, transplantation of, 1499
Autologous conjunctival transplantation, 1118
Autologous disk (ADCT), chondrocyte transplantation, 947
Autologous dopamine–secreting adrenal chromaffin cells, 1026
Autologous grafting, 1342
Autologous hematopoietic stem cells, 527
lentivirus-transduced, 502
Autologous IVD chondrocytes, 947
Autologous limbal stem epithelial cells, 1137
Autologous matrix-induced chondrogenesis (AMIC), 1503
Autologous mesenchymal progenitors, 1016–1017
Autologous mesenchymal stem cells (MSCs), 946, 1028–1029
Autologous NK therapies, 774
Autologous saphenous veins, 1525
Autologous somatic cells, 846
Autologous workflows, 1470, 1471*t*, 1472–1473
Automation in tissue engineering systems, 1490
Automobile manufacturing, 1469
Auxotonic systems, 599
avb3, 1316
Avulsive injury, self-inflicted gunshot wound, 1203*f*, 1204*f*
Axicabtagene ciloleucel, 770–771
Axon regeneration, 1049–1052, 1166, 1171

B

Bacon's method, 1587
Bacterial biofilm, 1221
Bacterial burden, 1333–1334
Bacterial colonization, 1333–1334
Bacterial-essential polynucleotides, 1310
Bacterial infections, 1310–1311
Baldness, 1299–1300
BALL-1 B-cell leukemia, 777–778
Barth syndrome, 611
Basal cell carcinoma, 1344*f*
Basal cells, 1260–1261, 1276
Basal lamina, invasion of, 81–82
Basement membrane-associated collagens, 123
Basement membrane (BM), 119, 217
microperforations, 228–230, 229*f*
Basic fibroblast growth factor (bFGF), 207, 864, 888, 948, 1213
Basic helix-loop-helix (bHLH)
activators, 148
factors, 148
family of transcription factors, 147
genes, 147
motif, 147
protein, 147–148
Basketweave pattern, 1318
Batten's disease, 7, 1028
Bayesian networks, 47
BBB. *See* Blood-brain barrier
B-cell lymphomas, 775–776
B cells, 365
Benign odontogenic neoplasm, right maxillectomy for removal of, 1206*f*
Bereitschaftspotential, 1039

Best corrected visual acuity (BCVA), 1145
Beta blockers, 1153, 1454
β-Catenin, 84, 240
β-cell
  challenge of making, 658
  destruction of, 657
  differentiation in vitro, 659
Beta-cyclodextrins (β-CD), 536
Beta galactosidase gene, 1289–1290
β-glycerophosphate, 888
β1 integrins, 1311, 1311*t*
Beta-thalassemia, 766–768
β-tricalcium phosphate scaffold, 1016–1017
Betaxolol, 1153
Bevacizumab (Avastin), 1147
B4G12, 1140
Biaxial rotating bioreactor, 179, 180*f*
Bicarbonate-based medium, 1461
Bicuspid aortic valves, 637
Biglycan, 990
Bilateral testicular agenesis, 873–874
Bile duct-derived organoids, 728
Biliary epithelial cells (BECs) *vs.* hepatocytes, 723–724
Biliary network engineering, 747
Biliary tissue engineering, 746–747
Bioactive compounds, 295
Bioactive factors, 825, 852, 938, 995–996, 995*f*, 999
Bioactive molecules, 826
  for in situ kidney regeneration, 836*t*
  morphogens as, 134–135
Bioactives, wound repair, 1322–1324
Bioartificial corneal equivalents, 1120
Bioartificial liver (BAL) devices, 738
Bioartificial muscle (BAM), 1359
Bioartificial organ, 1275*f*
Bioartificial pancreas
  antibiofouling approaches, 671
  beta-O_2 device, 668–669, 668*f*
  biocompatibility of encapsulation systems, 669–670
  extravascular devices, 668
  history of, 666
  macro- or microedevices, 667–669, 667*f*
  multilayer capsule approaches, 670–671
  pathogen-associated molecular patterns, 670
  replenishable cell sources and encapsulation, 666–667
Biobrane, 1339
Bioceramics, 921*t*
Biocompatibility, 866, 1052, 1102, 1167, 1208, 1221, 1234, 1473, 1512, 1561–1563
Biocompatible hydrophilic lipogels, 791
Biocompatible implant, 1120
Biocompatible materials, 1135, 1208–1209
Biocompatible polymeric scaffolds, 899
Biodegradability, 895, 1221, 1232–1234, 1397–1398
Biodegradable biomaterials, 850–854
  in medical implants, 319*t*
  natural collagen matrix, 851–854
    acellular tissue matrices, 852
    collagen, 852
    matrix binding with growth factors, 852–854
    silk fibroin (SF), 852
  synthetic scaffolds, 850–851, 851*t*
    biodegradable properties, 850–851, 851*t*
    porosity, 851
Biodegradable hydrogels, 758–759
Biodegradable polymers, 282, 979–980, 1074*t*
  selection criteria, 317–318
Biodegradable polyphosphazenes, 332
Biodegradable polyurethanes, 330–331
Biodegradable synthetic polymers, for 3D bioprinting, 1399
Biodegradable vicryl mesh, 1339
Bioengineered cornea, 1120
Bioengineered epithelial tissue equivalents, 1119
Bioengineered living skin equivalents, 1339–1340
Bioengineered skin constructs, 1331, 1338, 1348
  acellular constructs, 1331
  Apligraf, 1341–1344
  bioengineered living skin equivalents, 1339–1340
  cell-based constructs, 1331
  commercial considerations, 1336–1337
  components of skin and engineered skin equivalent, 1332*f*
  construct priming and didactic paradigm, 1347–1348, 1348*f*
  Dermagraft, 1341–1344
  dermal replacement, 1339
  dermis, 1332–1333, 1332*f*
  design considerations, 1335–1336, 1336*f*
  engineering skin tissue, 1335, 1335*f*
  epidermal regeneration, 1338
  epidermis, 1331–1332, 1332*f*
  FDA-approved indications
    cutaneous indications, 1340–1341, 1340*f*
    oral indications, 1341
  immunological considerations, 1338
  impaired healing and mechanisms, 1333–1335
    abnormalities at cellular level, 1335
    acute *vs.* chronic wound healing, 1333
    bacterial colonization, 1333–1334
    growth factor imbalances, 1334
    ischemia, 1334–1335
    matrix metalloproteinase activity, 1334
    moist wound healing in chronic wounds, 1334
  mechanisms of action (MOA), 1345–1347, 1347*f*
  process considerations, 1337
  regulatory considerations, 1337
  skin structure and function, 1331
  wound bed preparation, 1344–1345, 1346*f*
  wound healing process, 1333
Bioengineered tissue
  innervation in, 2
  vascularization in, 2
Biofabricated three-dimensional tissue models, 1419*t*, 1435
  biomaterials for, 1421
    cell selection, 1424–1425
    naturally derived biomaterials, 1421, 1423*t*
    synthetically derived biomaterials, 1422–1424
  brain and nerve tissue models, 1425–1427
  cancer models, 1427–1435, 1428*f*
    heart tissue models, 1428–1430, 1429*f*
    liver tissue models, 1430–1432, 1431*f*
    stroma tumor, 1428
    tumor metastasis, 1427–1428
    tumor microenvironment (TME), 1427
    vascular tissue models, 1432–1435, 1433*f*
  disease modeling, 1425–1435
  drug screening, 1425–1435
  extrusion printing, 1420–1421
  inkjet printing, 1418–1420, 1419*t*
    electromagnetic inkjet printing, 1419
    low-viscosity materials, 1418–1420
    piezoelectric inkjet printing, 1418–1419
    thermal inject printing, 1418
    water-based materials, 1418–1420
  light-assisted bioprinting, 1421
    digital light processing (DLP)–based bioprinting, 1421
    two-photon polymerization (TPP)–based bioprinting, 1421
  therapeutics and toxicology, 1425–1435
Biofabrication, 9–13, 10*f*, 24–25, 568–570, 569*f*, 924–925, 925*t*, 1274–1276, 1370, 1446–1447
  bioinks for, 982–985, 984*t*
  bioprinting techniques, 982, 983*f*
  cartilage tissue, 981–985
  computerized tomography scans, 981
  magnetic resonance imaging, 981
  scaffolds for, 981–982, 982*f*
Biofilms, 1333–1334
Biohybrid scaffold, 899
Bioinks, 295, 982, 996–997, 1396, 1418–1422, 1472
  biomaterials as
    biodegradable synthetic polymers for structure-based 3D bioprinting, 1399
    hydrogel-based bioinks for cell-based bioprinting, 1396–1399
    scaffold-free cell printing, 1399–1400
  for cartilage reconstruction, 982–985, 984*t*
  clinical application, 998–999
  G-code, 996
  hybrid, 13
  hydrogel scaffold, 996*f*
  inkjet printed graphene oxide–gelatin pattern, 997*f*
  ligament and tendon structures, 997–998, 998*f*
  photo-crosslinkable, 300
  polymer's degradation, biological effects of, 298–299
  properties, 299*f*
  shear thinning and viscosity, 996*f*
  SLA printed high-resolution microneedles, 997*f*

Biologically derived polymers, 318–326, 333
Biological scaffolds, 715–716
Biological systems, mechanical forces in, 237–238
Biological tooth replacement and repair, 1187
  dental-tissue regeneration, 1191–1196
    cell- and gene-based strategies, 1196
    control of specificity of, 1193–1194
    dental postnatal stem cells, 1194–1195
    directed tissue regeneration, 1195
    injury-regeneration balance, 1192–1193
    natural tissue regeneration, 1191–1192, 1192*f*
    signaling-based strategies, 1195–1196
    signaling events in, 1193
  tooth development, 1187–1189, 1188*f*
  whole tooth-tissue engineering
    bioteeth from cell-seeded scaffolds, 1189–1190
    cell sources, 1191
    root formation, 1190–1191
    stem cell-based tissue engineering of teeth, 1189
Biologic gels, 625
Biology of wound repair, 1310–1317
  extracellular matrix organization, 1316–1317
  granulation tissue, 1312–1316, 1313*f*
  inflammation, 1310
  reepithelialization, 1310–1312
  transition from inflammation to repair, 1310
  wound contraction, 1316–1317
Bioluminescence imaging (BLI), 761
Biomanufacturing for regenerative medicine
  academic clinical center, advancing at, 1478*t*
  approved products, 1474*t*
  cell banking advance, 1477
  challenges in, 1470–1472, 1471*t*
  closed-modular biomanufacturing system, 1476–1477
  current landscape of, 1469–1470
  current workflows for, 1470, 1471*t*
  medical applications, 1477–1478
  off-the-shelf product, 1477
  preservation advances, 1477
  regulatory challenges for, 1473–1476
    creating standard, 1475–1476
    Food and Drug Administration guidance document, 1474
    PHS Act 361, 1473
    21st Century Cures Act of 2016, 1473
  space exploration, 1478
  synthetic biology advance, 1477
Biomanufacturing processes, 1530, 1532
Biomaterial-driven strategies for bone regeneration, 921*t*, 922–923, 923*t*
Biomaterials, 2, 363–365, 569*f*, 850–851, 888, 897–898, 900–901, 919, 926–927, 940–942, 947, 989–990, 1007, 1052, 1093, 1103, 1394–1396, 1473, 1562
  as artificial extracellular matrices, 139–140
  biofabricated three-dimensional tissue models, 1421
    cell selection, 1424–1425
    naturally derived biomaterials, 1421, 1423*t*
    synthetically derived biomaterials, 1422–1424
  as bioinks, three-dimensional bioprinting
    biodegradable synthetic polymers for structure-based 3D bioprinting, 1399
    hydrogel-based bioinks for cell-based bioprinting, 1396–1399
    scaffold-free cell printing, 1399–1400
  bone tissue engineering, 1513–1516
    diaphyseal defects, regenerative repair of, 1513–1516, 1515*f*
  for cartilage reconstruction, 979–980
  cell-based three-dimensional (3D) renal constructs, 831*t*
  fabrication for repair, 1054–1058
  FDA-approved, 1562
  hematopoietic stem cells (HSCs), 758–759, 759*t*
  implantation, 1053–1054
  injectable, 2–4
  natural polymers, 1058–1072
    extracellular matrix polymers, 1058–1065
    polymers derived from the blood, 1071–1072
    polymers from marine/insect life, 1065–1070, 1068*f*, 1069*f*, 1070*f*
  nonallergenicity of, 1358–1359
  nontoxicity of, 1358
  selection of, 317–318
  semisynthetic, 112
  smart, 2–4, 3*f*
  synthetic polymers, 1072–1080, 1074*t*
    nonbiodegradable hydrogels, 1077–1080
    poly α-hydroxy acid polymers, 1073–1077
  tissue-derived, 54–55
  wound repair, 1322–1324
Biomaterials science, 1232–1236
  biomaterial-based immune modulation, 1233
  biomaterial redesign, 1235–1236
  classes of, 1233
    ceramic-based materials, 1235
    naturally derived polymers, 1233–1234
    synthetic polymers, 1234–1235
Biomechanical forces, 44
Biomimetic-driven strategies, growth factors, 923–924, 923*t*, 924*t*, 925*t*
Biomimetic materials, 322, 926
Biomimetic scaffolds, 297, 927, 1013
Biomimetic tracheal construct, 1409
Biomimetic vascular scaffold, 832
Biomimetic whole-organ lung bioreactor, 1281*f*
Biomimicry organ-on-a-chip models, 194
Biomolecules, 924, 943–944
  in bone tissue engineering, 925*t*
Bionic ear, 1406–1407
Biophotonics, 1375
Biophysical cues, 265–267
Biophysical stimulation, 1528–1529
Biopolymers, 1103
Bioprinted ear construct, 1407*f*
Bioprinted patches, 1528
Bio-printed scaffolds, 1301
Bioprinting technologies, 295, 1446
  inkjet three-dimensional, 12
  laser-assisted, 305
  quantum-on-demand, 569–570
  three-dimensional organ, 138
Bioprosthetic valve, 639–640
Bioreactor design principles, 179–180
  biaxial bioreactor design, 180*f*
  cell manufacturing quality attributes and process analytics technology, 189
  integration of multiple compartments, 193–194
  mimicking native environment
    cell environment, 186–188, 193
    mass transport, 181–184
    physiological biomimicry cues, 184–186
Bioreactors, 16–17, 25, 158–159, 477, 645, 833, 991–992, 1213–1214, 1236–1237, 1265–1266, 1370–1371, 1488, 1528–1529
  applications, 195–197
    drug testing and screening, 195–196
    experimental models of disease, 196
    prognostic/diagnostic tools, 196–197
  articular cartilage products, tissue engineering of, 1502–1503
  categories, 179
  for cultured meat production, 1371–1372
  Dermagraft, manufacture of, 1490
  design principles, 179–180
    biaxial bioreactor design, 180*f*
    cell manufacturing quality attributes and process analytics technology, 189
    integration of multiple compartments, 193–194
    cell environment, 186–188, 193
    mass transport, 181–184
    physiological biomimicry cues, 184–186
  development of, 2
  fluidized bed, 16–17
  food tissue engineering, 1361–1362
  high-aspect rotating vessel (HARV), 189–190
  industrial, 1379–1380
  living, 626
  lung tissue engineering, 1280–1281, 1281*f*
  macrobioreactors, 179–191
  microarray, 478*f*
  microbioreactors, 179, 191–197
  microgravity, 189–190
  perfusion, 16–17, 159–160
  real-time assessment in, 190–191
  rotating, 167
  RWV, 190
  scaling up of, 188
  sustainable, 188–189
  tissue culture, 166
  TransCyte, manufacture of, 1490
Bioregulatory molecules, in cell cultures, 183
Bioresorbable grafts, 625–626
Bioresorbable scaffolds, 896–897
Bioscaffolds, 926–927
BioSeed C, 1504

Biosheet technique, 459–462
Biosynthesis, fibrillar collagen, 121
Biotechnology, 1585
Bioteeth, 1189
  from cell-seeded scaffolds, 1189–1190
Biphasic calcium phosphate microparticles, 1403–1406
BIX 01294, 1097
Bladder and urethra
  biodegradable biomaterials, 850–854
    natural collagen matrix, 851–854
    synthetic scaffolds, 850–851
  cell sources, 846–850
    bladder and ureter cells, 846
    stem cell sources, 846–848, 847*t*
  clinical trials, 856–858
    clinical studies, 857–858, 857*f*
    clinical translation, 856–857
  fibrotic bladder model, 854–855
  mechanism of cell therapy
    cell expansion, 848
    multipotentiality, 848–849, 848*t*
    paracrine effects and immunomodulatory properties, 849–850
  preclinical models, tissue regeneration models, 854, 855*t*
Bladder atrophy model, 854–855
Bladder augmentation, 4–5
Bladder dystrophy model, 855
Bladder exstrophy, 463
Bladder-shaped biodegradable scaffold, 856–857
Bladder submucosa (BSM), 851–852
Blastocyst, 421–424
  fertilized, 423–424
  formation, 425
  hatched, 424
  inner cell mass (ICM) of, 467
Blastomere, 424
  single, 422–424, 423*f*, 428
Bleomycin, 1452
Blimp1, 1295
Blocking cell death pathways, 1096
Blood-brain barrier (BBB), 523, 1425–1426, 1540
Blood components from pluripotent stem cells
  megakaryocytes/platelets, 769–770, 769*f*
  red blood cells, 765–768
    beta-thalassemia, 766–767
    fetal hemoglobin, 766–767
    induced PSCs (iPSCs), 767
    Mi2beta, 767–768
    sickle cell disease, 766–767
  white blood cells, 770–779
    granulocytes, neutrophils, 778–779
    lymphocytes, NK cells, 773–775
    lymphocytes, NKT cells, 775–776
    lymphocytes, T cells, 770–773
    monocyte-derived dendritic cells, 776–777
    monocyte-derived macrophages, 777–778
Blood–gas barrier, 1273–1274, 1276
Blood islands, 1155
Blunt trauma, 1203
B lymphocytes, 65–66
Bmp gene, 809
Bmpr1a, 1296
BMPs. *See* Bone morphogenetic proteins
Body-on-chip systems, 2, 17–18, 196, 1544
  advanced in vitro modeling systems, 1444–1445
  Advanced Tissue-engineered Human Ectypal Network Analyzer (ATHENA) program, 1454
  Defense Advanced Research Projects Agency (DARPA), 1454
  drug development, 1445*f*
  Ex vivo Console of Human Organoids (ECHO), 1452–1454, 1453*f*
  integrated multiorganoid model systems, 1449–1455, 1450*f*
    additional disease modeling, 1452
    cancer, 1450
    drug testing/toxicology, 1450–1451, 1451*f*, 1452*f*
  National Institutes of Health, 1454
Bone applications
  BMP-2, 1209
  direct technique, 1211
  gene transfection, 1211
  growth factors, 1209–1210
  hemi-mandible, regeneration of, 1210*f*
  indirect delivery methods, 1211
  mesenchymal stem cells, 1211–1212
  minimally invasive surgery, 1209
  muscle-flap prefabrication technique, 1209–1210
  nonviral gene delivery systems, 1211
  oligo(poly(ethylene glycol) fumarate), 1212
  recombinant protein-based strategies, 1210–1211
  rhBMP-2, 1209–1210
  scaffolds, 1208–1209
  secondary-staged tissue-engineered graft, 1210*f*
  surface chemistry, 1212
Bone bioreactor, 261–264
Bone, cartilage, stromal progenitor (BCSP), 1011
Bone engineering, in vivo, 261–264
Bone formation, 261–262
Bone-implant design, 248–250
Bone implants
  osseointegration of, 249
Bone in vivo bioreactor, osteosynthesis in, 263*f*
Bone marrow (BM), 371–372, 463, 630, 681, 1190
  cell seeding, 630
Bone marrow–derived endothelial progenitor cells, 1262–1263
Bone marrow–derived mesenchymal stem cell (BM-MSCs), 829, 864–865, 885*t*, 886, 894, 948, 994–995, 1124, 1322
Bone marrow-on-a-chip, 760
Bone marrow stem cells (BMSCs), 846, 848–849, 999, 1010, 1016–1017, 1124, 1424–1425
Bone marrow stem cells, nonocular stem cells, 1124–1126
  endothelial colony-forming cells, 1125–1126
  hematopoietic stem cells/$CD34^+$ cells, 1124
  hematopoietic stem cells/$CD34^+$ cells and retinal disease, 1124–1125, 1125*f*
  mesenchymal stem cells, 1124
  potential for stem cells in ocular repair and tissue engineering, 1126
Bone marrow stimulation, 893
Bone marrow (BM) transplantation, 765
Bone matrix, 973–974
Bone morphogenetic protein (BMP), 18, 83–84, 219, 469, 693, 714, 806–807, 809, 894, 930, 995, 1007, 1009, 1013–1014, 1016, 1193, 1209, 1228, 1254–1255, 1296–1297, 1512–1513
Bone morphogenetic protein-2 (BMP-2), 894, 898, 901, 926, 930, 949*t*, 952, 1009, 1011–1014, 1016, 1209, 1215, 1512–1513
Bone morphogenetic protein-4 (BMP-4), 599, 806–807, 894, 1009, 1013–1014, 1190, 1211, 1539
Bone morphogenetic protein-6 (BMP-6), 886, 894, 929
Bone morphogenetic protein-7 (BMP-7), 809–810, 836*t*, 948
Bone morphogenetic protein-12 (BMP-12), 948, 949*t*
Bone morphogenetic protein-13 (BMP-13), 943–944, 952
Bone-muscle-flap prefabrication technique, 1209–1210
Bone regeneration, 348, 540, 1403–1406
  biomaterial-driven strategies for bone regeneration, 921*t*, 922–923, 923*t*
  bone biofabrication, 924–925, 925*t*
  cell types in, 920*t*
  clinical translation, 929–931
  ex vivo/in vivo small and large animal preclinical models, 926–929
  growth factors, biomimetic-driven strategies, 923–924, 923*t*, 924*t*, 925*t*
  self-reparative capacity of bone, 919–922, 922*f*
  skeletal stem cells, 917–919
  vascular bone, development of, 925–926
Bone tissue engineering, 897–898, 1511–1517
  augment endogenous bone regeneration, delivery of molecules and/or scaffolds to, 1512–1513
  biofactors, 1512
  biomaterials development and three-dimensional printing, 1513–1516
    diaphyseal defects, regenerative repair of, 1513–1516, 1515*f*
  and bone regeneration, 917, 918*f*
    biomaterial-driven strategies for bone regeneration, 921*t*, 922–923, 923*t*
    bone biofabrication, 924–925, 925*t*
    cell types in, 920*t*
    clinical translation, 929–931

ex vivo/in vivo small and large animal preclinical models, 926–929
growth factors, biomimetic-driven strategies, 923–924, 923*t*, 924*t*, 925*t*
self-reparative capacity of bone, 919–922, 922*f*
skeletal stem cells, 917–919
vascular bone, development of, 925–926
cells, 1511–1512
craniofacial defects, regenerative repair of, 1516–1517
scaffolds, 1512
Bone-to-bone interfaces, 164
Boston type 1 keratoprosthesis, 1167
Bottom-up approach, 897, 924, 996, 1395–1396
Bovine biphasic matrix, 1504
Bovine bone xenograft, 1209
Bovine mammary gland stem cells, 1363–1364
Bovine TMJ disk cells, 1213
Bowel tissue, 847–848
Bowman's capsule, 813, 832–833
renal stem cells in, 828
Bowman's membrane, 1135–1136
BPD. *See* Bronchopulmonary dysplasia
Brain and nerve tissue models, 1425–1427, 1426*f*
Brain–computer interfaces (BCIs), 1037, 1040
Brain-controlled wheelchair, 1041*f*
Brain-derived neurotrophic factor (BDNF), 1051, 1073, 1156–1157
Brain implants, 1026*f*
cell protection and regeneration implants, 1028–1030
cell implants secreting endogenous factors, 1028–1029
cell implants secreting engineered factors, 1029
controlled-release implants, 1030
encapsulated cell brain implants, 1029–1030
cell replacement implants, 1025–1028
cell line implants, 1027–1028
primary tissue implants, 1025–1027
disease targets for, 1031–1032
replacement and regeneration implants, 1030–1031
Brain–machine interfaces (BMIs), 1042
barriers to, 1042
context-aware, 1040–1041, 1041*f*
for controlling devices, 1038*f*
mutual learning, 1040
restoration of grasping, 1040*f*
signals, 1037–1038
voluntary activity *versus* evoked potentials, 1038–1040
Brain organoid, 1540–1541, 1541*f*
Brain signals, 1037–1038
Brain tissue repair, 269
Branching morphogenesis, 217
basis of, 217–218
extracellular matrix components in, 226–228
in kidney, 222–224
in lung, 218–220, 220*f*
mathematical and computational models, 230–231
geometry, 230
mechanical forces, 230
microRNAs in, 225–226
in salivary gland, 220–222, 221*f*
signaling mechanisms, 230–231
Breakthrough Devices Program, 1563
Breakthrough therapy designation, 1564*t*, 1565, 1565*t*
Breast
anatomy and development, 557–558
cell types and related challenges, 560–561
reconstruction, 558–565
Breast cancer, 84, 557, 1448–1449
diagnosis and treatments, 558
in silico, 570–571
modeling, 565–570
spheroids, 1401–1403
Breast cells, 98
Breast tissue engineering, 560
Breast tissue test systems, 566–570
Brimonidine, 1153
Brn3a, 1154–1155
Brn3b, 1154–1155
Bromodeoxyuridine (BrDU), 761, 849–850, 1289–1290, 1292–1293
Bronchoalveolar stem cells (BASCs), 1261
Bronchopulmonary dysplasia (BPD), 458
Bronchospheroid models, 1541
Bruch's membrane, 1145–1146, 1148–1150
BST Cargel (chitosan), 1503
BTBD7, 221–222
Bulge as stem cell source, hair follicle stem cells, 1291–1292, 1292*f*
characteristics of, 1292–1294
molecular signature, 1293–1294, 1293*t*
multipotency, 1294
quiescence, 1292–1293
Burosumab, 930
X-linked hypophosphatemia, 930
Bursa omentalis, 409
Business issues, 1573
aging population, 1573–1575, 1576*f*
exemplary chronic health conditions, 1575*f*
product development, 1577–1580, 1577*f*
direct reprogramming of differentiated cells, 1580
embryonic stem cells, 1578–1579
induced pluripotent stem cells, 1579–1580
small molecule-induced differentiation, 1580
regenerative medicine, risk of, 1575–1577
reimbursement, 1580–1582

C

Cabimer protocol, 659
Cadherins, 71, 80
cytoplasmic tail of, 71
homophilic binding of, 240
Caenorhabditis elegans, 147, 240–241
Caisson disease, 930
Calcific aortic valve disease (CAVD), 1430
Calcification, 639
Calcium alginate film, 1234
Calcium channel blockers, 1097
Calcium hydroxide, 1196
Calcium influx, 1094, 1097
Calcium ions, 71
Calcium phosphate ceramics, 1015
Calcium phosphate granules, 1208–1209
Calcium phosphates (CaP) cement, 901, 1234–1235
Calcium polyphosphate, 944
Calcium sensing receptor (CaSR), in marrow-derived stem cells, 263–264
Calcium sulfate, 1235
Calmodulin (CaM), 1094
Calvarial defect, 927
Calvarial model, 1010
cAMP. *See* Cyclic adenosine monophosphate
CAMs. *See* Cell adhesion molecules
Cancer, 871–872, 891, 1028, 1542–1543
biofabricated three-dimensional tissue models, 1427–1435, 1428*f*
heart tissue models, 1428–1430, 1429*f*
liver tissue models, 1430–1432, 1431*f*
stroma tumor, 1428
tumor metastasis, 1427–1428
tumor microenvironment (TME), 1427
vascular tissue models, 1432–1435, 1433*f*
cancer, 84, 557, 1448–1449
diagnosis and treatments, 558
in silico, 570–571
modeling, 565–570
spheroids, 1401–1403
integrated multiorganoid model systems, 1450
morbidity, 248
Cancer metastasis, 248, 1449
mechanical processes involved in, 249*f*
Cancer-on-chip models, 196, 1448–1449
Candida albicans, 1300
Canonical EPU model, 1291, 1302
Canonical Wnt pathway, 582*f*, 809, 815, 894
Capillary leak syndromes, 1260
Cap mesenchyme (CM), 809, 812
Capsid, 527–529
CAR. *See* Coxsackie adenovirus receptor
Carbon dioxide, 1361–1362, 1461, 1463–1464
Carbonic anhydrase (CAN), 790
Carbonic anhydrase inhibitors, 1153
Carbon nanotubes (CNTs), 472–473
Carboxylated agarose (CA), 266–267
Carboxylic acid, 758–759
Cardiac-chimeras (CCs), 586
Cardiac development, with pluripotent stem cells, 581–582
Cardiac differentiation, of human stem cells, 599–601
Cardiac fibroblasts, reprogramming, 584–585
Cardiac fibrosis, 609–611
Cardiac muscle, 148
Cardiac neural crest (CNC), 579
Cardiac organoid, 1539–1540
Cardiac organs-on-chip, 13

Cardiac patches, 1527–1529
  bioprinted patches, 1528
  contractile patches, 1528–1529
  electromechanical integration, h, 1528–1529
  noncontractile, 1527–1528
  vascularization, 1528
  vascular integration of, 608
  vasculature in, 608
Cardiac progenitor cells (CPCs), 585–586, 1429–1430
  in vivo fate mapping of, 582
Cardiac regeneration, 594, 612
  in neonatal heart, 584*f*
Cardiac repair, neonatal, 582–583, 585
Cardiac resident mesenchymal stem cells (CMSCs), 584–585
Cardiac stem cells, 586
  origin of, 579–581
Cardiac tissue and heart valves, 1408
Cardiac tissue engineering, 598*f*
  artificial scaffolds, 602–604
  Barth syndrome, 611
  biophysical cues, 604–606, 605*f*
    electrical stimulation, 604
  biophysical stimulation, 599
  cardiac fibrosis, 609–611
  cardiomyocytes derivation, 599–601
  cell source, 594–598
  chronic hypertension, 611
  clinical problem, 593–594
  decellularization approach, 601–602
  design parameters for, 595*t*
  diabetes-related cardiomyopathy, 611
  directed cardiac differentiation, 599–601
  engineered heart tissue, 606–608
  engineered heart tissue models, 609
  mechanical stimulation, 604–606
  modeling of disease, 609–612
    patient-specific cardiomyocytes, 609, 610*f*
  perfusion, 606
  as platform for pharmacologic studies, 611–612
  scaffolds, 598–599, 601–604, 603*f*
  stem cell-derived cardiomyocytes, purification and scalable production, 601
  titin mutation-related dilated cardiomyopathy, 611
  vascularized cardiac patches, 608
  in vivo applications of, 606–609, 607*f*
Cardiac toxicities, 437–438
Cardiac valves, 1430
Cardiomyocytes (CMs), 451, 473–474, 480, 579–580, 585, 593–594, 601, 1521–1524, 1539
  derivation of, 599–601
  electrical coupling of, 608–609
  generation of, 600*f*
  maturation of, 184
  metabolic demand of, 599
  stem cell-derived, purification and scalable production of, 601
Cardiomyopathy
  diabetes-related, 611
  titin mutation-related dilated, 611
Cardiovascular diseases (CVD), 593–594, 617, 1369–1370, 1428–1429
Cardiovascular tissue-engineered medicine products
  approval and commercialization, pathway to, 1530–1532, 1530*f*, 1531*t*
  cardiac patches, 1527–1529
    bioprinted patches, 1528
    contractile patches, 1528–1529
    electromechanical integration, h, 1528–1529
    noncontractile, 1527–1528
    vascularization, 1528
  clinical situation/reality, 1521
  clinical trials of, 1523*t*
  components for, 1521–1525
    cell sources, 1521–1524
    scaffolds, 1524–1525
  considerations for, 1521, 1522*f*, 1522*t*, 1523*t*
  valves, 1526–1527
    current valve prostheses, 1526
    tissue-engineered valves, 1527
  vascular grafts, 1525–1526
    autologous saphenous veins, 1525
    scaffold-based TEVGs, 1526
    scaffold-free grafts, 1526
  whole heart, 1529–1530
Cardiovascular tissues, 157
Cares1S, 1503
Carotenoids, 1147
Carticel, 892–893, 980
Cartilage Autograft Implantation System (CAIS), 1503
Cartilage defect
  of patella, 1506*f*
  pathophysiology, 1499–1500
  surgical treatment options for, 1500
Cartilage-derived stem/progenitor cells (CSPCs), 887*f*
Cartilage matrix and cell injuries, 970
Cartilage monitoring and real-time control, 1463–1464, 1464*f*
Cartilage oligomeric matrix protein (COMP), 888
Cartilage reconstruction
  biofabrication of cartilage tissue, 981–985
    bioinks for, 982–985, 984*t*
    bioprinting techniques, 982, 983*f*
    computerized tomography scans, 981
    magnetic resonance imaging, 981
    scaffolds for, 981–982, 982*f*
  biomaterials for, 979–980
  cell sources for, 980
  elastic cartilage, 979
  fibrocartilage, 979
  hyaline cartilage, 979
  osteochondral tissue engineering, 985
Cartilage repair, 475
  fiber-reinforced constructs for, 162–163
  stratified and osteochondral constructs for, 163–164
Cartilage tissue engineering, 1212–1214
  mesenchymal stem cells (MSCs), 891–897
    cells for, 892–897
    chondrogenesis, 893–895
    factors influencing outcomes of, 896–897
    general properties of, 892
    scaffolds for, 895–896
  scaffold, 163*f*
Cartilaginous endplates, 938
Cartimaix, 1503
Cartipatch, 1505
CARTISTEM, 1505
Caspase-3, 955
Caspase-dependent pathways, 1095
Caspase-independent pathways, 1095
Caspase inhibitors, 967
Catalase (CAT), 787–788
Catalytically dead Cas9 (dCas9), 435
Cataracts, 1145
Cathepsins, 1095
Cationic b-CD-based polymers (bCDPs), 536
Cationic block copolymer gene delivery, 954–955
Cationic liposomes, 1211
Cationic macromer poly(ethylene imine), 1211
Cationic polymers, 533–534, 536
Cation-induced gelation, 286
Caveolae, 524–525
CavME, 524–525
CBERCDRH, 1561
Cbfa1/Runx2 expression, 897
CCL2, 287–288
CD4 T cells, 772
CD8 T cells, 772
CD28, 770–771
CD31 *(Pecam-1)*, 1258
CD31 antibodies, 833–835
CD80, 849–850
CD86, 849–850
CD90 (Thy1), 1011
CD105, 1011, 1196
$CD133^+$ cells, 828
CDH. *See* Congenital diaphragmatic hernia
CDRH Device Advice, 1554
Ceiling culture, 1360, 1377
Celastrol, 1098
Cell types, 75–76
Cell adhesion, 350
  and function, 282
  to vitronectin, 126
  and water-in-air contact angle, 281*f*
Cell adhesion molecules (CAMs), 71–72, 72*f*
Cell-adhesion peptides, 850–851
Cell- and gene-based strategies, 1196
Cell- and tissue-based therapies for lung disease
  bioengineering of lung tissues, 1265–1266
    conducting airways, tissue engineering for, 1266
    extracellular matrix in lung structure and repair, 1265–1266
    mesenchymal stromal cell products, 1265
    mesenchymal stromal cells, 1265
    pulmonary macrophage transplantation, interstitial lung disease, 1266
  challenges for, 1253, 1254*f*

lung morphogenesis, regeneration, 1254–1263, 1255*f*, 1256*f*
diverse epithelial and mesenchymal cell types, 1256–1257
embryonic development of alveolar capillaries, 1258–1259, 1259*f*
endothelial progenitor cells in lung repair, 1262–1263
lung epithelial progenitor/stem cells, 1260–1262
lung microvasculature in lung repair, 1262
pulmonary vasculature, structure and function of, 1257–1258, 1258*f*, 1260*f*
signaling and transcriptional pathways, lung formation, 1256
pulmonary cell-replacement strategies for lung regeneration, 1263–1265
induced pluripotent stem cells, to pulmonary epithelial cell lineages, 1264–1265
induced pluripotent stem cells, treatment of pulmonary disease, 1263–1264, 1264*f*
Cell arrays, 476–477
Cell banking advance, 1477
Cell-based approaches, 1239–1242
delivery strategies in, 1239, 1240*t*
scaffold-based cell delivery, 1241–1242
scaffold-free cell delivery, 1239–1241
Cell-based bioprinting, 1391, 1396, 1399
Cell-based constructs, 1331
Cell-based gene therapy, 493
Cell-based products, 1560–1561
in medical product testing, 1568
Cell-based therapies, 403, 585–588, 1145, 1577
diabetic retinopathy, 1148*t*
Ggaucoma, 1148*t*
retinal degenerative diseases, 1148*t*
Cell-based tissue engineering of kidney, 826–835
cell sources, 826–830, 834*t*
tissue-engineered cellular three-dimensional renal constructs, 830–835
Cell behaviour, 133–134
effect of polymer chemistry on, 280–284
influence of surface morphology on, 284–285
patterned surfaces to control, 285
Cell-biomaterial constructs, intervertebral disk (IVD) regeneration, 940–945
annulus fibrosus repair and regeneration, 942–944, 942*f*, 943*f*
composite cell-biomaterial intervertebral disk implants, 944–945, 945*f*, 946*f*
nucleus pulposus cell-biomaterial implants, 940–942
Cell–cell adhesion, 79, 240
changes in, 80
Cell–cell interactions, 470
in collectives, 243–244
during tissue morphogenesis, 136
Cell–cell signaling, 317
Cell chambers, 195
Cell characterization, 1555
Cell communication, 136
Cell community effect, 1191
Cell-containing cryopreserved human valve allografts, 1527
Cell culture, 65–66, 75, 76*f*
bioregulatory molecules in, 183
in vitro, 275–278
three-dimensional *versus* two-dimensional, 475
Cell cycle, 75*f*
Cell determination, 145–146
Cell differentiation, 145–146, 146*f*, 244–245
Cell division, 76*f*
Celleextracellular matrix adhesion (CelleECM), changes in, 80–81
Cell encapsulation techniques, 286, 866, 1240*t*
Cell–extracellular matrix interactions, 96–104, 106*f*
adhesion and migration, 96–98
apoptosis, 100
differentiation, 99–100
proliferation, 98–99
Cell fate
commitment, 40–41
stem, 38, 40
Cell-free tissue engineering of kidney
in situ kidney regeneration, 835, 836*t*
stromal cell–derived factor-1, 837
Cell-generated forces, 247–248
Cell implants
endogenous factors, 1028–1029
engineered factors, 1029
Cell interactions
with fibronectin, 96–97
with polymers, 275–285
in suspension, 286
in vitro cell culture methods, 275–278
in vivo methods, 278–280
with three-dimensional polymer scaffolds and gels, 287
unique to in vivo setting, 287–289
Cell-level monitoring, tissue engineering systems, 1463
reporter-based gene expression imaging, 1463, 1463*f*
Cell line brain implants, 1027–1028
Cell lines
immortalized, 406–407
next-generation, 407
Cell markers, 1196
Cell–material interaction, 301
Cell matrices, axonal regrowth, 1177–1178
Cell–matrix interactions, 93, 97, 317
Cell-mediated cytotoxicity, 1338
Cell-mediated immune responses, 1338
Cell-mediated tension, 125
Cell microenvironment, 193
Cell migration, 244*f*
rates of, 282–283
Cell motility
mechanisms, 81
stimulation of, 81
Cell necrosis, 181
Cell nucleus, 65–68
Cell patterning, 482
Cell pellets, 1239–1241, 1240*t*
Cell-penetrating peptides (CPPs), 535
Cell polarity, changes in, 81
Cell–polymer interactions
high-throughput methods for characterization of, 278
Cell protection and regeneration brain implants, 1028–1030
cell implants secreting endogenous factors, 1028–1029
cell implants secreting engineered factors, 1029
controlled-release implants, 1030
encapsulated cell brain implants, 1029–1030
Cell proliferation, 187*t*, 244–245
Cell quality, 1503
Cell reactivation, 947
Cell receptors
of commonly used viral-based gene therapy vectors, 498*t*
ligands, 523
Cell rejection, transplanted, 404
Cell replacement brain implants, 1025–1028
cell line implants, 1027–1028
primary tissue implants, 1025–1027
Cells
as building units in tissue engineering, 138–139
in culture, 76*f*
morphogenesis of, 194
in tissues and organs, 76–78
from umbilical cord, 458
Cell scaffolds, 1230–1231, 1561
Cell-seeded technology, 644–645, 851, 854, 864–865
as implants, 1211–1212
biomaterial scaffold, 166–167
bioteeth from, 1189–1190
endothelial, 623–624
Cell selection, 1196, 1424–1425
Cell sheet engineering technology, 865, 994, 1239, 1240*t*
Cell sorting techniques, 1028
Cell sources, 4–8, 5*f*, 15*f*, 22–23
cartilage reconstruction, 980
cell number requirements, 743–744
fetal and adult progenitors, 744
immortalized cell lines, 744
primary cells, 744
tissue organoid models and applications, 1537–1538
whole tooth-tissue engineering, 1191
Cell-specific gene RPE65, 1125
Cell-specific promoters, 437
Cell-specific targeting strategies, 502–505
Cell spheroids, 14, 14*f*
Cell–substrate adhesions, 240–241
Cell-supporting phase, 162
Cell surface, 71
markers, 437
receptors, 94
Cell survival, and differentiation, 111–112

Cell therapy, 6–7, 22–23, 630, 1527
  age-related macular degeneration (AMD), 1147–1149
  clinical studies, 947–948
  development of, 1554
  extracellular matrix (ECM) for, 744–746
    modifications in scaffold chemistry, 745–746
    natural scaffold chemistry and modifications, 745
    porosity, 746
    synthetic scaffold chemistry, 745
  preclinical studies, 946–947
  skeletal tissue engineering, 1010–1013
Cell transplantation, 973
Cell transplants, 559
Cell transport, and vascularization, 345
Cell types, of epithelial layer, 709–710
Cellular affinity, 561
Cellular apoptosis, 167
Cellular biology, of heart valves, 636–637
Cellular core, 1030*f*
Cellular differentiation, nature's approach to, 260–261
Cellular dynamics, machine learning of, 47
Cellular encapsulation technologies, 661
Cellular material, 559
Cellular mechanosensing, 238–243
Cellular medicament, 661
Cellular nutrition, 404–405
Cellular organization
  nature's approach to, 260–261
  three-dimensional architecture and, 742–743
Cellular polarity, defined, 81
Cellular receptors, for lentivirus vectors, 501–502
Cellular scaffolds, 560–565
Cellular spheroids, 14
Cellular uptake, 523–526
Cellulose, 323
Cellulose nanofibers, 998
Cellurization in vivo, 457
Cell viability, 1408
  strategies to maintain, 659–661
Cementogenesis, 1224, 1227
Cementum, 1224
Center for Biologics Evaluation and Research (CBER), 1337, 1473, 1553, 1561–1562
Center for Devices and Radiological Health (CDRH), 1487, 1553, 1561–1562
Center for Drug Evaluation and Research (CDER), 1487, 1494, 1553
Centers for Medicare and Medicaid Services (CMS), 1494, 1581–1582
Central auditory prostheses, 1102–1103
Central nervous system (CNS), 452, 1027
Cerebral organoids, 1425–1426
Cervical cord, 1052–1053
Cervical thymus, in mouse and human, 688–689
CFTR. *See* Cystic fibrosis transmembrane conductance regulator
CGMP. *See* Current good manufacturing practice
Channelrhodopsin-2 (ChR2), 1174–1176
Characterized chondrocyte implantation, 1504
Chemical degradation, 352
Chemically induced liver progenitors (CLiPs), 730–731
Chemistry, manufacturing and controls (CMC), 1555, 1558
Chemoattractants, potent, 620
Chemokines, 101–102
Chemotactic factors, 1316
Chemotherapy anticancer agent cisplatin (CDDP), 1094–1096
Chemotherapy drug resistance, 1543–1544
CHI3L1, 1154
Chiari malformation, 461
Chicken flu, 1369–1370
Chimeric antigen receptors (CARs), 770–771, 771*f*, 773–775, 779
  T cell therapies, 770–772, 775, 1470
Chinese hamster ovary cells, 1470
CHIR99021, 830
Chitin, 325
Chitosan, 325, 535–536, 563, 940–942, 1053*t*, 1059–1065, 1069, 1208–1209, 1234
  nanoparticles, 535–536
  scaffolds, 1070*f*, 1233
  and stem cell transplantation, 1070*f*
Cholangiocytes, 725–726, 727*f*
Cholinergic muscarinic agonists, 1153
Chondral defects, 1499–1500
Chondral fissures, 970
Chondral injuries, 970–971, 970*t*, 973–974
Chondral repair, 971–973
Chondral tears, 970
ChondroCelect, 980, 1504
Chondrocyte-laden hydrogel bioink, 1394
Chondrocyte-laden type II collagen scaffold, 979–980
Chondrocytes, 287, 319, 887–889, 892–893, 899, 921–922, 967, 969–973, 979, 983–984, 1009, 1406, 1500–1504, 1506
Chondrofiller, 1503
Chondrogenesis, 886, 888, 893, 898–899, 980, 1406–1407
  chondrogenic potential, 893–894
  signaling in, 894–895
Chondrogenic progenitor cells, 972
ChondroGide, 1503
Chondroinductive growth medium, 893
Chondroitin sulfate, 984–985
Chondroitin sulfate proteoglycans (CSPGs), 1051
Chondron, 1505
Chondroprogenitor cells, 893
Chondrotissue, 1503
Chorioallantoic membrane (CAM) assay, 97
Choroid plexus cells, 1030
Chromaffin cells, 286
Chromatin, 67–68
Chromatin state, 1256
Chromosomal DNA, 67–68
  of target cells, 495
Chronic bladder diseases, 847–848
Chronic contusion/compression, 1053–1054
Chronic diseases and impairments, 1574–1575
Chronic electrical stimulation, 1098–1099
Chronic granulomatous disease (CGD), 778
Chronic hypertension, induced left ventricle hypertrophy, 611
Chronic inflammation, 111
Chronic kanamycin-treated mouse model, 1095
Chronic kidney disease (CKD), 1, 812–813, 825–828
Chronic leg ulcers, 1320
Chronic liver disease, 737, 743
Chronic liver failure, 737
Chronic liver injury, 743
Chronic lung diseases, 1261–1262
Chronic obstructive pulmonary disease (COPD), 1253, 1257, 1265–1266, 1274
Chronic periodontitis, 1229
Chronic rejection, 1493–1494
Chronic wounds, 1317–1318, 1323, 1333, 1341–1342, 1493, 1495
  healing, 1309, 1333
  moist wound healing in, 1334
  trials with, 1492–1493
Chyle, 557–558
Ciliary neurotrophic factor (CNTF), 1156–1157, 1172
Ciprofloxacin, 1103
Circulating angiogenic cells (CACs), 1125
Circulating immune cells, 1262
Circulatory system, 137
Cited1, 809
c-Jun NH2-terminal kinase (JNK), 1095
CKD. *See* Chronic kidney disease
c-kit, 1196
c-KIT-positive EPCs, 1262–1263
CLARITY, 16
Clark electrode sensor, 1461
Clathrin-mediated endocytosis (CME), 524
Cleft formation, in mammalian lung, 219*f*
Clinical attachment levels (CAL) of tooth, 1221
Clinical disorders, 157
Clinical investigator responsibilities, 1567
Clinical reality, from mathematical models to, 47–48
Clinical research
  conducted outside of United States, 1568
  involving children, 1566
Clinical studies, in regenerative medicine, 54
Clinical translational pathway, 54–58
CLIP. *See* Continuous liquid interface printing
CLiPs. *See* Chemically induced liver progenitors
Closed-modular biomanufacturing system, 1476–1477
Clotbuster drugs, 1172
Clustered regularly interspaced short palindromic repeats (CRISPR), 450, 453, 507, 1100
  gene editing, 659–661
Clusters of differentiation 34 (CD34), 1295
Ciliary neurotrophic factor, 1028
CME. *See* Clathrin-mediated endocytosis

CMs. *See* Cardiomyocytes
CMSCLCs, 584–585
CNC. *See* Cardiac neural crest
CNNs. *See* Convolutional neural networks
CNTs. *See* Carbon nanotubes
Coagulative necrosis, 593–594
Cochlear Corporation, 1101–1102
Cochlear fluids, local delivery to, 1103
Cochlear implants, 1178–1179
Cochlear nucleus implants, 1102–1103
Cochlear prostheses, 1098–1099, 1101, 1103
  fully implantable, 1101–1102, 1102*f*
Coculture methods, 1338
Coculture organoid model of bronchial epithelial cells and lung fibroblasts, 1541
Codes of Federal Regulation (CFR), 1473–1474
Collagen, 72, 120–123, 226–227, 319–320, 459–460, 562, 758–759, 831–832, 831*t*, 851–852, 854–855, 864, 892, 897–898, 941*t*, 968, 971, 979, 984*t*, 990, 993, 1138–1139, 1208–1209, 1211–1214, 1231–1234, 1257, 1265–1266, 1314–1315, 1317–1318, 1323, 1359, 1378, 1398, 1404*t*, 1423*t*, 1462, 1484, 1491, 1495
  deposition of, 410–411
  fibrillar, 121–122, 211
  fibrils, 121
  fibrous, 135–136
  scaffolds, 865, 1058–1059, 1059*f*, 1501–1502
  synthesis, 121*f*
  types, 120*t*
Collagen-1-based scaffolds, 927
Collagenases enzymes, 1317
  Collagenase-2, 1314
  Collagenase-3, 1314
Collagen-based inert matrix, 858
Collagen-binding domain (CBD), 864
Collagen-binding domain–vascular endothelial growth factor (CBD–VEGF)-bound collagen, 852–854, 864
Collagen bundles, collection of, 1317
Collagen-coated porous silk protein scaffold, 865
Collagen fibers, 635–636, 938–940, 943, 990
Collagen fibrils, 207, 210, 895, 970–971
Collagen-glycosaminoglycan sponge, 1339
Collagen-hyaluronic acid, 947
Collagen hydrogels, 837, 1015
Collagen-induced fibroblast migration, 108
Collagen IV, 227, 1358
Collagen matrix, 1450
Collagen matrix Mucograft, 1214
Collagen meshwork, 1372–1373
Collagenous capsule, 288
Collagen remodeling, 1317
Collagen–vitrigel scaffold, 831–832
Collagen XVIII, 227
Colloid osmotic pressure (COP), 789–790
  viscosity and, 789–790
Colon tumor model, 1450
Colony-forming units (CFUs), 40
Colored 3D printer, 1512
Combination scaffolds, 564
Committed progenitor (CP) model, 1291
Common lymphoid progenitors (CLPs), 770
Common myeloid progenitor (CMP), 767
Compaction Aggregation (CoROC), 684
Composite cell-biomaterial intervertebral disk implants, 944–945, 945*f*, 946*f*
Composite constructs, 1513
Composite grafts for mucocutaneous junctions, 1214–1215
Composite scaffolds, 898, 922–923
Composite tissues, 1409
  oral and maxillofacial surgery, 1215
Compression, 238
Computational fluid dynamic techniques, 183
Computational models, 37
Computational molecular phenotyping, 1164
Computational simulations, 183
Computed tomography, 1209–1210
Computer-aided design (CAD), 295, 1209–1210, 1391–1392
Computer-aided manufacturing (CAM), 1391–1392
Computer-assisted 3D printing, 1057*f*
Computer-assisted design, 1056
Computer-controlled impact contusion injuries, 1052–1053
Computerized tomography, bone and cartilage reconstruction, 981
Conditional chimeric antigen receptors, 771–772
Condrocytes, 947–948
Conducting airways, 1266, 1276–1277
Conduits
  arteries, 618
  patency and failure, 618–619
  veins, 618
Condylar chondrocytes, 1212–1213
Congenital airway anomalies, 462–463
Congenital and acquired genitourinary tract, 875–876
Congenital anomalies, 457
Congenital bilateral absence of the vas deferens (CBAVD), 874
Congenital diaphragmatic hernia (CDH), 459, 461
Congenital facial clefts, 1203
Congenital glaucoma, 1153
Congenital heart disease, 462, 637–638
Congenital hereditary endothelial dystrophy, 1139
Congenital high airway obstruction syndrome, 462–463
Conjugated linoleic acid (CLA), 1363
Conjugation, of macromolecules, 531
Conjunctival epithelial stem cells, 1120
Conjunctival transdifferentiation, 1118
Connective tissue growth factor (CTGF), 948, 949*t*
Connexin 43, 1118–1119
Contaminants, pyrogenic, 670
Context-aware brain–machine interfaces (BMIs), 1040–1041, 1041*f*
Continuous liquid interface printing (CLIP), 305–306
Continuous projection printing methods, 1056
Contractile patches, 1528–1529
Controlled-release implants, brain, 1030
Contusion injuries, 1053–1054
Conventional scaffold-based fabrication method, 1396
Convolutional neural networks (CNNs), 46
Cooper's ligaments, 557–558
Coping behavior, 1364
Copolymers, 282, 327–329, 327*f*
Col2 promoter-driven luciferase, 1463
Cord blood (CB), 1156
Cord cells, umbilical, 643–644
Cornea decellularization, 1138
Corneal blindness, 1135
Corneal diseases, characterization of, 1137*t*
Corneal endothelial cells (CECs), 1119–1120, 1139–1140
Corneal epithelial cell, 283
  plasticity, 1117
Corneal epithelial stem cell markers, pursuit of, 1117–1118
Corneal fungal ulcers, 1138
Corneal replacement tissue, 1135, 1136*f*
  corneal anatomy and structure, 1135–1136
  endothelium, 1139–1140
  epithelium, 1135–1138
  markers for corneal cell types, 1137*t*
  stroma, 1138–1139
Corneal stem cells, 1115–1119
  corneal epithelial cell plasticity, 1117
  corneal epithelial stem cell markers, pursuit of, 1117–1118
  epithelial stem cells, 1115–1116
  limbal stem cell niche, 1116–1117
Corneocytes, 1289–1291
Coronary artery disease, 593–594
Cortical neurorganoids, 1543
Cortical thymic epithelial cells (cTEC), 681, 683, 687–688, 695
Corticosteroid dexamethasone, 1097
Costal and ankle chondrocytes, 1212–1213
Coxsackie adenovirus receptor (CAR), 497
CPPs. *See* Cell-penetrating peptides
Craniofacial bone, 1212
  platelet-rich plasma (PRP), 1210
  in vivo models for, 1208–1209
Craniofacial defects, 1016
  regenerative repair of, 1516–1517
Craniofacial tissue engineering, 1210–1211
Cre (CrePR1) construct, 1294
Cre/lox-based approach, 1150–1151
Cre-loxP and CRISPR technology, 1100
Crest cells, neural, 581
Crigler–Najjar syndrome type I, 740
CRISPR. *See* Clustered regularly interspaced short palindromic repeats
CRISPR-associated protein 9 (Cas9), 435, 450, 453, 508, 1171, 1543
CRISPR/dCas9 system, 955

Critical process parameters (CPP), 189
Critical quality attributes (CQA), 189
Critical size defect (CSD), 1010, 1014–1015, 1215–1216, 1511
Crohn's diseases, 1124
Crosslinkable alginates, 940–942
Cryopreservation, 559, 1477, 1491–1492, 1495
Cryopreserved allografts, 1321
Cryopreserved homograft, 639–640
Cryovein, 1525
Cryovessels, 622
Crypt base columnar (CBC) cells, 710
Cryptorchidism, 872
CSF2Ra, 1266
CSF2Rb, 1266
c-SRC, 1008
Ctnnb1, 809–811
Cultured autologous keratinocyte grafts, 1320
  advantage, 1320
  disadvantage, 1320
Cultured epithelial autograft (CEA), 1338
Cultured keratinocyte allografts, 1320
Cultured meat technology, 1369–1370
  benefits of, 1372
  biophotonics, 1375
  history of, 1372
  industrial production, challenges and requirements for, 1375–1382
    associated dangers and risks, 1381
    atrophy and exercise, 1380
    consumer acceptance and perception, 1382, 1383*t*
    fields, 1380
    industrial bioreactors, 1379–1380
    meat processing technology, 1381
    muscle fibers, tissue engineering of, 1378
    regulatory issues, 1381–1382
    safe differentiation media, muscle cells, 1377–1378
    safe media for stem cells, 1377
    scaffolds, 1378–1379
    senescence, 1381
    stem cell lines from farm-animal species, 1376–1377
  market for, 1382–1383
  media in publicity, 1382
  nanotechnology, 1375
  need and advantages of, 1370–1371
  organ printing, 1375
  scaffolding techniques, 1372–1373, 1373*f*
  self-organizing tissue culture, 1373–1375
Cultured oral mucosal cells, 1119
Culture medium, 75–76, 767, 946
  development for biomanufacturing, 1472
  formulation and growth characteristics of bone marrow–derived MSC, 885*t*
  pH, 1461
  in publicity of cultured meat, 1382
  TransCyte, manufacture of, 1489–1490
Cumulus–oophorus complexes (COCs), 866
Current good manufacturing practice (cGMP), 60, 1080, 1470, 1475*t*, 1559
Current Procedural Terminology (CPT) codes, 1581
Cutaneous epithelial stem cells, 1289
  epidermal stem cells, 1301–1302
    as therapy, 1302
    tissue engineering with, 1301–1302
  epithelial stem cells in aging, 1298–1299
  hair follicle stem cells, 1291–1295
    bulge as stem cell source, 1291–1292
    ectodermal appendages, 1295–1297
    marker expression, subpopulations by, 1294–1295
    skin homeostasis, 1297
    wound healing, 1297–1298
    wound-induced hair follicle neogenesis and regeneration, 1298
  interfollicular epidermal stem cells, 1289–1291
    epidermal proliferative unit *vs.* committed progenitor, 1290–1291
    label-retaining cell (LRC) methods, 1291–1292
  skin as active immune organ, 1300–1301
    cross talk between hair follicles and immune system, 1300–1301
    inflammatory memory of skin cells, 1301
  stem cells in alopecia, 1299–1300
Cutting-edge testing methods, 1557
C-X-C chemokine receptor 4 (CXCR4), 837
CXCL8, 101–102
Cyanobacteria, 1370
Cyclic adenosine monophosphate (cAMP), 73–74
Cystectomy, 856
Cysteine, secreted protein acidic and rich in, 210–212
Cystic fibrosis, 521, 1543
Cystic fibrosis rectal organoid model, 1543–1544
Cystic fibrosis transmembrane conductance regulator (CFTR), 450–451
Cystic spheroids, homogenous differentiation of, 476
Cytodex-3 microcarrier beads, 1378
Cytokeratins, 240–241, 1260–1261
Cytokine release syndrome (CRS), 770–771
Cytokines, 365, 620, 636, 758, 759*t*, 773–774, 883, 888, 971, 1007–1008, 1013–1014, 1230–1231, 1236, 1238–1239, 1317, 1319–1320
Cytoplasm, 68–72
Cytoskeleton, 69–71, 239
Cytosolic calcium homeostasis, dysregulation of, 1094
Cytotoxic cross-linking mechanisms, 300
Cytotoxic T lymphocytes (CTLs), 773

D

Dacron, 619–620, 622, 1525
Damaged oligodendrocytes, 1050–1051
DAMPs. *See* Danger associated molecular patterns
Danger associated molecular patterns (DAMPs), 672–673, 673*f*, 1310
DDR2-mediated migration, 108
DDRs. *See* Discoid domain receptors
Deafness, definition of, 1093
Debridement, 985
Decellularization (DC), 8–9, 24, 745, 825, 834*t*, 1399, 1527
  of native tendon/ligament tissue, 993
  of tissues and organs, 864
Decellularized dental pulp, 1195
Decellularized extracellular matrix (dECM), 13, 186, 745, 1065, 1067*f*, 1361, 1423*t*, 1524–1525
Decellularized extracellular matrix-based bioinks, 1409–1410
Decellularized nucleus pulposus (NP), 940–942
Decellularized organs, 24
Decellularized scaffolds, 1274–1281
Decellularized tissues, 460
dECM. *See* Decellularized extracellular matrix
Decorin, 990
Dedifferentiated fat (DFAT), 1377
Deep brain stimulation (DBS), 1025
Defense Advanced Research Projects Agency (DARPA), 1454
Deformable microcarrier beads of edible (nonanimal) material, 1378
Degenerative diseases of temporomandibular joints (TMJs), 1202–1203
Degradable polymers, 282
Degradation
  proteoglycan, 94
  rate, 646
Delivery, definition of, 1564
Delta ligand 1 (DL1), 772
Denatured collagen, 1311
Dendritic cells (DCs), 364–365, 681
  immune system, 1332
  monocyte-derived, 776–777
  thymic, 681
Dendritic epithelial T cells (DETCs), 1299
Denosumab, 930
De novo liver engineering, 267–268
De Novo marketing authorization, 1563
DeNovo Natural Tissue (NT) Graft (DeNovo NT), 1503
Dental caries, 1192–1193
Dental follicle stem cells (DFSCs), 1222–1225, 1227*t*
Dental implants, 1187
Dental plaque biofilm, 1192–1193
Dental postnatal stem cells, 1194–1195
Dental primordia, reorganization of, 1189–1190
Dental pulp stem cells (DPSCs), 1193–1194, 1222–1223, 1225, 1227*t*
Dental-tissue regeneration, 1191–1196
  cell- and gene-based strategies, 1196
  control of specificity of, 1193–1194
  dental postnatal stem cells, 1194–1195
  directed tissue regeneration, 1195
  injury-regeneration balance, 1192–1193
  natural tissue regeneration, 1191–1192, 1192*f*
  signaling-based strategies, 1195–1196
  signaling events in, 1193

Dentin–pulp complex, 1191–1195
Dermagraft, 1321, 1339–1344, 1474*t*, 1483–1484, 1487–1489, 1494–1495
  design concepts for, 1487, 1488*t*
  for diabetic ulcers, 1493
  manufacture of, 1489–1490, 1489*f*
    bioreactor design, 1490
    cells, 1489
    medium, 1489–1490
  production processes, 1490–1492
    distribution and cryopreservation, 1491–1492
    problems with commercial culture, 1492
    release specifications, 1491
  to treat gingival recessions, 1341
  vasculitis, treatment of, 1344*f*
Dermal fibroblasts, 447*f*, 643, 980
Dermal papilla (DP), 1291
Dermal replacement, 1339
Dermal substitutes, 1309
Dermis, 1332–1333, 1332*f*, 1335–1336
Dermomyotome, 146
Descemet's membrane (DM), 1135–1136
Desmosomes, 240, 1332
Detoxification devices, 1422
Developmental engineering, 264–265
Device-associated infections, 1514
Dexamethasone, 888, 1103, 1360
dHAND, 148
Diabetes mellitus, 657, 1139, 1369–1370
Diabetic foot ulcers (DFUs), 1317–1318, 1321–1322, 1342
Diabetic macular edema, 1155
Diabetic neuropathic foot ulcer, 1340*f*
Diabetic retinopathy, 1124–1125, 1155, 1166
  cell-based therapies, 1148*t*
  stem cell–based therapies, 1155–1156
Diabetic ulcers, 1493
Diacylglycerol (DAG), 73–74
Dialysis, 620
Diaphyseal defects, regenerative repair of, 1513–1516, 1515*f*
Dickkopf-related protein 1 (DKK1), 599, 897
Die3 chip, 1168*f*
Diet, 1095
Diethylaminoethyl (DEAE)-dextran, 286
Differentiated cells, 994
Digital Imaging and Communications in Medicine (DICOM) format, 1391–1392
Digital light processing (DLP), 981–982, 1419*t*, 1421, 1426–1428
  -based 3D bioprinter, 1430–1431
Digital micromirror device (DMD) chip, 1395, 1421
Digital ulcers, 1348*f*
DILI. *See* Drug induced liver injury
Diltiazem, 1097
Directed tissue regeneration, 1195
Direct pathway of transplant rejection, 1493–1494
Direct perfusion bioreactors, 1379–1380
Direct reprogramming of differentiated cells, 1580
Direct technique, oral and maxillofacial surgery, 1211
Discoid domain receptors (DDRs), 96
Discoidin domain receptor (DDR) 2, 211–212
Discoid lupus erythematosus, 1299
Disease modeling, 435–436, 448–450, 1301
  challenges and future possibilities in, 450–451
Disease-modifying potential, of induced pluripotent stem cells, 451–452
Disk cells, 947–948
Dispenser system, 1392
Distal airway engineering, 1276–1277
Distraction-assisted in situ osteogenesis, 1209–1210
Distraction osteogenesis, 1008–1010, 1016–1017
  bone regeneration, 1513–1514, 1513*f*
  with Ilizarov devices, 1511
    growth factors associated with, 1514
Disulfide-bonded polypeptides, 72–73
Diver's disease, 930
Diverse epithelial and mesenchymal cell types, 1256–1257
Division of Manufacturing and Product Quality (DMPQ), 1559
Division of Regulatory Project Management in OTAT, 1557
DLP printing. *See* Dynamic light processing printing
d-methionine, 1096–1097
DNA
  chromosomal, 67–68
  methylation, 68
  replication, 68
DNA-binding domain, 67
DNAM-1, 773–774
DNA methyl transferase enzymes, 68
DNase, 833
Docosahexaenoic acid, 1150
Dominant disease, vision enhancement systems, 1173
Donor antigens, 405
Donor epithelial cells, 1119
Dopaminergic neurons, 1029–1031
Double-chamber bioreactor, 899
Double-labeling technique, 1294
Double negative (DN)
  cells, 681–682
  thymocyte progenitors, 681–682
Double-stranded RNA (dsRNA), 519
Doxorubicin, 1428, 1449
Drosophila trachea, 218
  budding in, 218*f*
Drug analysis, tissue organoid models and applications, 1543–1544, 1543*f*
Drug delivery system, 1234–1235
  vision enhancement systems, 1172
Drug development, body-on-a-chip, 1445*f*
Drug-eluting nano- and microspheres, 1073–1076
Drug-induced fibrogenesis, 1431–1432
Drug-induced hearing loss, 1096
Drug induced liver injury (DILI), 737, 741, 1430–1431
Drug product (DP), 1558
Drugs
  carriers, 521–522
  discovery, 436–438, 1400–1401
  immunosuppressive, 421
Drug screening, 1301, 1403, 1444
  in liver and cardiac organoids, 1452*f*
  three-dimensional tissue models, 1425–1435
Drug substance (DS), 1558
Drug testing/toxicology, 1450–1451, 1451*f*, 1452*f*
Dry age-related macular degeneration (AMD), 1578–1579
Dry electrodes, 1042
dsRNA. *See* Double-stranded RNA
Dua's layer, 1135–1136
Ductus arteriosus (DA), 462
Dulbecco's modified Eagles medium (DMEM), 885*t*, 886, 1189–1190, 1489
Dynamic bioreactor culture, 179
Dynamic cell seeding, 644
Dynamic light processing (DLP) printing, 305–306
Dynamic reciprocity, 93

E

Early-stage development, regulatory process, market, 1554–1557
  chemistry, manufacturing and controls, 1555
  clinical, 1556–1557
  pharmacology and toxicology, 1555–1556
EBB. *See* Extrusion-based bioprinting
E-box, 147
EBP. *See* Elastin-binding protein
EBs
  effect of, 476
  formation of, 472, 476, 476*f*
  maturation of cardiac function in, 475
Ebselen, 1095–1096
E-cadherin, 80, 82, 228, 1312
EC angiogenesis, 1280
Eccrine glands, 1296
Ecdysone, 507
Echocardiography, fetal, 462
ECM. *See* Extracellular matrix
ECoG. *See* Electrocorticography (ECoG)
ECs. *See* Endothelial cells
Ectoderm, 428, 806, 1187
Ectodermal appendages, hair follicle stem cells, 1295–1297
  nail, 1296–1297
  sebaceous glands (SGs), 1295–1296
  sweat glands, 1296
Ectomesenchyme, 1187–1189
Ectopic grafts, 743
Ectopic hair cells, 1100
Ectopic transplantation assays, 917–918
Eculizumab (Soliris), 1147
Edentulous bone loss, 1201–1202
Edge effect, 1346–1347, 1347*f*
Edmonton protocol, 665

EDPCs. *See* Epicardial derived progenitor cells
EEGs. *See* Electroencephalogram (EEG)
EG cells. *See* Embryonic germ cells
EGF. *See* Epidermal growth factor
eHAND, 148
Ejaculation, definition of, 874–875
Ejaculatory system, male reproductive organs, 874–875
  engineering vas deferens, 874
  spinal ejaculation generator, 875
Elastic cartilage, 560, 979
Elastic fibers, 126, 990
Elastin, 72, 320, 1257, 1265–1266
Elastin-binding protein (EBP), 95–96
Elastin fibers, 635–636
Elastin-like polypeptides (ELPs), 940–942
Electrical coupling, of cardiomyocytes, 608–609
Electrically conducting polymers, 284, 348
Electrically elicited electroretinogram (eERG), 1178, 1179*f*
Electrically elicited VEPs (eVEPs), 1178–1179
Electrically evoked compound action potentials (eCAPs), 1178
Electrical signals, 185–186
Electrical stimulation, 348–349, 602, 604, 1169, 1380
  visual prostheses, 1167–1170, 1168*f*
Electrocorticography (ECoG), 1037–1040
  advantages, 1038
60-electrode array (Orion), 1170
Electro ejaculation, 875
Electroencephalogram (EEG), 1037–1038, 1040, 1042, 1587
Electromagnetic inkjet printing, 1419
Electromechanical integration, h, 1528–1529
Electronic common technical document (eCTD), 1558–1559
Electronic interfaces to conduct messages, 1052
Electroporation, 471, 1176
Electroretinogram (ERG), 1149
Electrospinning, 9–12, 11*t*, 980, 1015–1016, 1058, 1076, 1379, 1396
Electrospun-aligned PCL nanofibrous scaffold, 899–900
Electrospun biodegradable fibers seeded with amniotic-derived mesenchymal progenitor cells, 1016
Electrospun keratin scaffold, 995–996
Electrospun nanofibrous scaffold, 895*f*
Electrospun short nanofibers, 997–998
Elevated blood sugar, 1155
Elevated intraocular pressure (eIOP), 1153
ELISA, 1462
Embden–Meyerhof–Parnas, 791
Embryogenesis, 1051, 1262–1263, 1298, 1310–1311, 1322–1323
  of craniofacial skeleton, 1201
Embryoid bodies (EBs), 1540–1541
  formation, 426
Embryonic fibroblasts, mouse, 425
Embryonic germ (EG) cells, 5–6
Embryonic stem cells (ESCs), 5–6, 22–23, 37, 150–151, 160–161, 188, 407, 421, 436, 445, 457–458, 467, 581, 658, 776, 815, 826, 829–830, 833–835, 846, 865, 1010, 1027–1028, 1101, 1115, 1137, 1189, 1254–1255, 1376, 1381, 1424–1425
  business issues, 1578–1579
  clinical use of, 471
  coculturing, 477
  culture of, 475
  differentiation of, 470, 472
  directed differentiation, 471
  enrich specific lineages from human, 479*t*
  human embryonic stem cells (hESCs), 421
    approaches to, 421–425
    culture, 426
    derivative for regenerative medicine purposes, 426–428
    derivatives of, 421
    directed differentiation, 426–430
    early stages of, 423*f*
    maintenance of, 425
    morphology and markers of, 422*f*
    naive, 424
    safety concerns, 430–431
    spontaneous differentiation of, 427*f*
    subculture of, 425–426
  immune response, 481–482
  intrinsic property of, 471
  isolation of specific progenitor cells from, 479–480
  maintenance of, 468–471
  microenvironmental cues, 472–475
  mouse, 424–425, 445
  multiple, 470
  potential barrier to using, 509
  primed, 424
  in retinal regeneration
    photoreceptors from, 1122–1123
    retinal ganglion cells from, 1123
    retinal pigment epithelial from, 1121–1122
  self-renewal of, 468–470
  spontaneous differentiation of, 473
  transplantation, 480–482
  vascular differentiation of, 474*f*
Embryonic tooth primordium, 1189
Embryonic valvular interstitial cells, 636
Embryonic wounds, 1309
Emdogain, 1229–1230
EMTs. *See* Epithelial-to-mesenchymal transitions
Emulsion electrospinning, 9–10
Enamel matrix derivative (EMD), 1221, 1229–1230
En bloc harvesting, 1205–1206
Encapsulated cell brain implants, 1029–1030
Encapsulated cell technology (ECT), 1172
Encapsulated hemoglobin, 791
Encapsulation, 404, 661, 666, 666*f*
Endochondral ossification process, 893–894
Endocrine effects, 1377–1378
Endocrine organs, 1253
Endocytosis, 523–524
  fluid-phase, 523
  of nonviral vectors, 523
  receptor-mediated, 523
Endoderm, 806
Endogenous biomolecules, 1461
Endogenous bone marrow–derived mesenchymal stem cell (BMSC), 994–995
Endogenous gene regulation, 955
Endogenous ocular stem cells
  bioengineered cornea, 1120
  conjunctival epithelial stem cells, 1120
  corneal stem cells, 1115–1119
    corneal epithelial cell plasticity, 1117
    corneal epithelial stem cell markers, pursuit of, 1117–1118
    epithelial stem cells, 1115–1116
    limbal stem cell niche, 1116–1117
  endothelial stem cells, 1119–1120
  Müller stem cells, 1121
  retinal pigment epithelium (RPE) cell, 1121
  retinal progenitor cells, 1120–1121
  stromal stem cells, 1119
Endogenous photoreceptors, 1150–1151
Endogenous stem cells, 1099–1100
Endogenous tissue regeneration, recruitment of host cells for, 1238*f*
Endometrium, 864
Endomucin (Emcn), 265–266
Endoplasmic reticulum (ER), 1094
Endoplasmic reticulum stress, 1094
Endothelial cells (ECs), 100, 262–263, 266–267, 593, 875–876, 1258
  aortic, 205–206
  proliferation of, 267
  seeding, 623–624
Endothelial colony-forming cells (ECFCs), 1123–1126, 1263, 1398–1399
  therapeutic uses of, 1126
  vascular repair and vasoregenerative function of, 1126
Endothelial dystrophy, 1139
Endothelial islands, 621
Endothelial progenitor cells (EPCs), 473, 837, 1124–1125
  in lung repair, 1262–1263
Endothelial seeding into lung scaffolds, 1278–1279
Endothelial stem cells, 1119–1120
Endothelial-to-mesenchymal transformation (EMT), 636
Endothelial vessel networks, 1379–1380
Endothelium, 621, 1139–1140
Endotoxins, 670
End-stage kidney disease, 805
End-stage renal disease (ESRD), 825
Engelbreth–Holm–Swarm (EHS), 93
Engineered cartilage constructs, 159–160
Engineered cartilage integration, 160*f*
Engineered dermal constructs, 1321
Engineered epidermal constructs, 1320–1321
Engineered heart tissue (EHT) approach, 160–161, 1528

Engineered multilayer ovarian tissue model, 866
Engineered skin substitutes, 1321–1322
Engineered tissues, 2, 564–565
  with gene delivery, 538
  morphogenesis, 138–141
  vascularization of, 564–565
  viral delivery to, 538–539
Engineering cardiac tissue, design principles and components, 594–599
Engineering functional tissues, 158–159
  biological and mechanical requirements, 159
  criteria for, 159
  critical aspects of, 157
  guidelines, 158
  key challenges in, 158
  mechanical conditioning, 158–159
  parameters for, 158–159
  scaffolds, 158–159
    bioactive, 164–165
    bioinductive, 164–165
    design for engineering cardiac tissue, 166*f*
  in vitro platforms for high throughput screening of drugs and agents, 160–161
  in vitro studies, 157*f*
    culture parameters, 161–171
    to tissue engineering and regenerative medicine, 159–160
Engineering skin tissue, 1335, 1335*f*
Engineering viral vectors, 528–530
Enhanced green fluorescent protein (eGFP), 535–536
Enhanced meat, 1363
Enhanced myocardial mitotic activity, 587*f*
Enhanced permeation and retention (EPR) effect, 521–522
Enteric nervous system (ENS), 714
Enterocytes, 709–710
Enteroendocrine cells, 709–710
Entombing, 1191–1192
Enzyme-linked receptors, 73
Eosinophils, 364
Epac agonists, 1280
EPCAM. *See* Epithelial cell adhesion molecule
Eph receptor B3 (EphB3), 1259
Ephrin B2 *(Efnb2)*, 1258–1259
Ephrin receptor B4 (Ephb4), 1258
Epicardial cells, 581
Epicardial derived progenitor cells (EDPCs), 580–581
Epicel (Genzyme Tissue Repair), 1320
Epidermal/dermal substitutes, 1309
Epidermal growth factor (EGF), 94, 208, 888, 1214
Epidermal growth factor receptor (EGFR), 220–221, 713
Epidermal proliferative unit (EPU), 1289–1291
Epidermal regeneration, 1338
Epidermal stem cells, 1301–1302
  as therapy, 1302
  tissue engineering with, 1301–1302
Epidermis, 1289, 1331–1332, 1332*f*, 1335–1336, 1338, 1340
  by autologous transgenic epidermal culture, 1302
  consists of, 1289
  hair follicles (HFs), 1289
Epidermolysis bullosa (EB), 1342, 1344*f*, 1493
Epigenetic approaches, 1191–1192
Epigenetic memory, 1579
Epigenetic modification, 1097, 1256
Epigenetic remodeling, 446
Epilepsy, 1025, 1031–1032
Epinephrine, 1454
Epithelial barrier, 710
Epithelial cell adhesion molecule (EPCAM), 683
Epithelial cells, 79–81, 217, 709
  corneal, 283
  intestinal, 709–710
  vs. mesenchymal cells, 80*f*
  thymic, 682–685
Epithelial layer
  cell types of, 709–710
  intestinal, 709–710
Epithelial–mesenchymal transition (EMT), 79–83
  induction, model for, 85
  induction of, 86*f*
  molecular control of, 83–85
  transcriptional program, 82–83
  transcription factors, 82
    posttranscriptional regulation of, 83
Epithelial polarity, 81
Epithelial progenitor cells, 1116
Epithelial stem cells, 1115–1116
  in aging, 1298–1299
Epithelial tissues, 237
Epithelial-to-mesenchymal transitions (EMTs), 579, 646
Epithelium, 77–78, 217, 1135–1138
EPR effect. *See* Enhanced permeation and retention effect
ePTFE grafts, 619–620, 628*f*
ErbB signaling, 713
Erectile dysfunction (ED), 871, 876–877
  stem cell therapy for, 876–877
Error-related potentials (ErrPs), 1038–1039
Erythrocruorins, 792–793
Erythrocytes. *See* Red blood cells (RBCs)
Erythromelalgia, 451
Erythropoiesis, 767
Erythropoietin (EPO), 767, 826, 836*t*
Eschar, 1311
Escherichia coli, 852–854, 1403
ESCs. *See* Embryonic stem cells
eSight 3, 1166–1167
Esophageal atresia, 461–462
Esophagus, 461–462
ES-sac culture method, 767–768
Ethical issues, 1585–1586
  contextual factors, 1588–1589
  deconstruction, perils of, 1588
  duty and healing, 1587
  embrace brokenness, 1586
  intellectual property, 1590
  practical wisdom, 1587
Ethidium homodimer-1, 301–302
Eukaryotic cells, 65
European Medicines Agency (EMA), 776, 1530–1532, 1568
Evoked potentials, 1038–1040
Ewing sarcoma, 774
Excitation-contraction coupling, 171
Excitotoxicity, protection from, 1098–1099
Exocytosis, 523–524
Exogenous biomolecules, 1461
Exogenous fibroblast growth factor-2, 1014
Exogenous mesenchymal stem cells, 900–901, 1119
Exogenous scaffolds, 1400
Exogenous stem cells, 1093, 1101, 1263–1264
Exon skipping, 519–520
Exosomes, 536, 585, 890, 995–996, 1116
  stem cell–derived, 1230
Expanded polytetrafluoroethylene (ePTFE) grafts, 619
Expedited review programs, 1564–1565, 1564*t*, 1565*t*
External ear, 1406–1407
Extracellular matrix (ECM), 2–4, 8, 24, 72–73, 79, 119, 133–134, 179, 205, 217, 240–243, 343, 635–636, 830, 850, 864, 871, 888, 919–921, 938, 952, 968, 979, 990–992, 994, 1051, 1060*t*, 1149, 1309, 1312–1314, 1316, 1331, 1340, 1391–1392, 1403–1406, 1446, 1459, 1464, 1485, 1486*t*, 1524, 1528
  amount/levels, 1462
  bioregulatory components of, 1446
  for cell therapies, 744–746
  cell signaling, 229–230
  components, 119, 459
    in branching morphogenesis, 226–228
    degradation of, 224
  composition, 93–94
  decellularized, 186, 1525
  degradation, 229–230, 1311–1312
  ECM-derived hydrogels, 1452–1453
  ECM-mimicking biomaterials, 1221
  functions, 119
  glycoproteins, 135–136
  historical background, 93
  hypertrophic scars, characteristics of, 1319*t*
  keloids, characteristics of, 1319*t*
  ligands, 94
  in lung structure and repair, 1265–1266
  modulates morphogens release and signaling, 135*f*
  normal scars, characteristics of, 1319*t*
  organization, 1316–1317
  perfusion decellularization, 1525
  polymers, 1058–1065
  proteins, 4, 24, 119, 278, 476
  receptors, 94–96, 95*f*
  remodeling of, 217
  scarless regeneration, characteristics of, 1319*t*
  structure, 1462
  synthesis, 119
Extracellular milieu, 205

Extracellular vesicles (EVs), 365, 585, 1141
Extravascular macrocapsules, 659–661, 668–669
Extrinsic cytokines, 765
Extrusion-based bioprinting (EBB), 12–13, 297, 304–305, 1056, 1394, 1396–1397, 1404*t*, 1406–1408, 1429–1430, 1432–1435
  advantage of, 1394
  hydrogel-based bioinks, 1396–1397
  limitations to, 1420–1421
  poly(ε-caprolactone) (PCL), 1399, 1422
Ex vivo Console of Human Organoids (ECHO), 1452–1454, 1453*f*
Ex vivo gene therapy, 494–495, 1028–1029, 1103, 1196
  vs. in vivo gene therapy, 494
Ex vivo/in vivo small and large animal preclinical models, 926–929
Ex vivo perfusion and ventilation, 1263
Ex vivo strategy, 494–495
Eya1, 807
Eye, stem cells in
  endogenous ocular stem cells, 1115–1121
    bioengineered cornea, 1120
    conjunctival epithelial stem cells, 1120
    corneal stem cells, 1115–1119
    endothelial stem cells, 1119–1120
    Müller stem cells, 1121
    retinal pigment epithelium (RPE) cell, 1121
    retinal progenitor cells, 1120–1121
    stromal stem cells, 1119
  nonocular stem cells, 1121–1126
    bone marrow stem cells, 1124–1126
    embryonic stem cells in retinal regeneration, 1121–1124
    induced pluripotent stem cells (iPSCs) in retinal regeneration, 1121, 1122*f*
  ocular stem cells, 1116*f*

F

Fab@Home system, 306
Fabricating diseased organoids, 1543
Fabrication methods, 351, 897, 993, 1391, 1400–1401
Fabrication of fiber oriented AF tissue constructs, 943*f*
F-actin bundle arrays, 1317
Factor VIII antibodies, 875–876
Factor XIIIa-heparin bidomain peptide, 1071–1072
FAK. *See* Focal adhesion kinase
Fas-ligand- and TRAIL-mediated methods, 775–776
Fast track designation, 1564–1565, 1564*t*, 1565*t*
Fat-derived stem cells, 1485
Fat, tissue engineering of, 1359–1361
  adipocyte differentiation, biochemistry of, 1360
  cells, 1359–1360
  scaffold and cell seeding, 1360–1361
FBGC. *See* Foreign body giant cells
FBR. *See* Foreign body reaction
FDA-approved HDAC inhibitor vorinostat (SAHA), 1095, 1097
FDA Good Laboratory Practices (GLP), 55
FDA's Center for Biologics Evaluation and Research regulates biologic, 1473
FDA's Regenerative Medicine Advanced Therapy, 777–778
FDM. *See* Fused deposition modeling
Federal Food, Drug, and Cosmetic Act (FDCA), 1473, 1563
Feedback-based mechanisms, 38
Female infertility, 863
Female reproductive organs, 863
  neovagina reconstruction, 866–867
  ovarian follicles, tissue engineering, 866
  ovary, 865–866
  uterine cervix tissue engineering, 865
  uterus, 863
    acellular tissue engineering, 864
    cell-seeded scaffolds, 864–865
    scaffold-free approaches for, 865
  vagina, 866
Femoral head repair, bioprinting, 985
Femtosecond pulses, 1176
Fenton-type reactions, 1096
ferrylHb, 787–788
Fertility, 863
Fetal and adult stem cells, 826
  amniotic fluid stem cells, 829
  mesenchymal stem cells, 829
Fetal bovine serum (FBS), 1472
Fetal echocardiography, 462
Fetal fibroblasts, 1319–1320
Fetal germline, potential risk to, 386–387
Fetal hemoglobin (HbF), 766–767
Fetal immune system, 460
Fetal implantation, 461
Fetal liver stem cells, 724
Fetal mesencephalic grafts, 1031
Fetal progenitors, 744
Fetal stem cells, 457, 848
Fetal thymic organ culture (FTOC), 684
Fetal tissue, 461–462
Fetal tissue dopamine grafts, 1026–1027
Fetal tissue engineering, scaffolding specifics in, 459–460
FGF. *See* Fibroblast growth factor
Fgf8, 810–811
FGFR. *See* Fibroblast growth factor receptor
Fiber-deposited method, 980
Fiber-reinforced hydrogel, 162
Fibers
  collagen, 635–636
  elastic, 126
  elastin, 635–636
  meshes, 287
  reinforcement, 162–163
  sensory, 137–138
  stress, 239
Fibril-associated collagens with interrupted triple helices (FACIT), 121–123
Fibrillar collagen, 121–122, 211, 970–971
  biosynthesis, 121
Fibrillins, 126
  mutations, 126
Fibrillogenesis, 121*f*, 122–123, 125, 128
Fibrils, 121, 979
  axial and radial growth of, 121–122
  collagen, 121
Fibrin, 831*t*, 953, 971, 1053*t*, 1071–1072, 1072*f*, 1118–1119, 1138, 1234, 1241, 1311, 1315–1316, 1398–1399, 1404*t*, 1423*t*
Fibrin clot, 136, 1324
Fibrin-fibronectin clot, 100
Fibrin/fibronectin-laden wound space, 1312–1314
Fibrin/fibronectin-rich wound, 1316
Fibrin glue, 563
Fibrin hydrogel, 1379
Fibrinogen, 350, 1311, 1334, 1347–1348, 1398–1400
Fibrin-rich clot, 1310
Fibroblast-derived induced pluripotent stem cells, 1123
Fibroblast growth factor (FGF), 84, 94, 806–807, 809–810, 894, 1009, 1014, 1121–1122, 1209, 1277, 1313–1314, 1540
  signaling, 691
Fibroblast growth factor 1 (FGF-1), 1316
Fibroblast growth factor-2 (FGF-2), 620, 629, 886, 894, 980, 1014, 1316
Fibroblast growth factor-7 (FGF-7) (keratinocyte growth factor 1), 1485
Fibroblast growth factor 8 (FGFf8), 689–691
Fibroblast growth factor 10 (FGF10), 1277
Fibroblast growth factor receptor (FGFR), 94, 222*f*
Fibroblast-laden poly-caprolactone-based scaffolds, 184–185
Fibroblast-like cells, 1360
Fibroblast-like clonogenic cells, 917–918
Fibroblasts, 14, 103–104, 285, 319, 448, 560, 585*f*, 593, 1050–1051, 1214, 1239, 1312–1315, 1313*f*, 1317, 1321, 1333, 1338, 1399–1400, 1483–1485, 1486*t*, 1489, 1495
  3D in vitro cancer model, 1401–1403
  apoptosis of, 104
  migration, 1323
Fibrocartilage, 971, 979
Fibrochondrocytes, 952, 1212–1213
Fibronectin, 72–73, 97, 123–125, 208–209, 228, 350, 1053*t*, 1071, 1071*f*, 1311, 1314–1316, 1319, 1323, 1484
  accumulation of, 219
  cell binding to, 123–124
  cell interactions with, 96–97
  inhibition of, 228
Fibronectin-immobilized positron emission tomography (PET) scaffolds, 760
Fibronectin–integrin interactions, 99–100
Fibrosa, 635–636
Fibrosis, 288–289, 669–670
  cardiac, 609–611
  cystic, 521

Fibrotic bladder model, 854
Fibrous collagens, 135–136
Fibrous scaffolds, 895
Fibula osteocutaneous vascularized free flap, 1207*f*
Ficoll/Percoll, 886
Fiducials, 1032
Filaments, intermediate, 239
Filler material, 1232
First heart fields (FHF), 579–580
Fisher's equation, 42–43
Flavopiridol, 1073–1076
Floating embryoid body-like culture, 1121
Fluid flow-induced shear stress, 470
Fluidized bed bioreactors, 16–17
Fluid-phase endocytosis, 523
Fluid pressurization, 892
Fluid shear, 238
Fluorescence-activated cell sorting (FACS), 473, 479, 814–815
Fluorescently labeled albumin, 1447–1448
5-fluorouracil (5-FU), 1448–1451, 1454
Fluosol-DA, 793–794, 794*f*
FN functional domains, 1323
Focal adhesion kinase (FAK), 104–105, 228, 1008, 1315
Focal adhesion kinase-dependent mechanotransduction, 1008
Focal adhesion kinase (FAK)/Src, 105–107
Focal adhesions, 240, 243
  tension sensing at, 242*f*
Follicle atrophy, 1298–1299
Follistatin, 836*t*
Food and Drug Administration (FDA), 1487, 1512–1513
  FDA–licensed tissue-engineered medicine products, 1522*t*
Food and Drug Administration Modernization Act of 1997 (FDAMA), 1563–1565
Foodborne illnesses, 1369–1371
Food tissue engineering, 1355–1356
  advantages, 1361
  challenges, 1361
  consumer acceptance, 1364–1365
  efficiency, 1362
  enhanced meat, 1363
  fat, tissue engineering of, 1359–1361
    adipocyte differentiation, biochemistry of, 1360
    cells, 1359–1360
    scaffold and cell seeding, 1360–1361
  greenhouse gas (GHG) emission, 1356
  meat, tissue engineering of, 1357*f*
  medical application, 1356–1357
    function, 1356–1357
    uniqueness, 1356
  scale, 1361–1362
  skeletal muscle, tissue engineering of, 1357–1359
    cells, 1357–1358
    gel and seeding, 1358–1359
  taste, texture, juiciness, 1362–1363
Forces of distraction, 1008
Foreign body giant cells (FBGC), 279, 282, 288
Foreign body reaction (FBR), 278–279, 363, 366, 410–411, 411*f*
  development of, 279*f*
  role of geometry in, 411–412
  tuning chemical composition to prevent attachment, 412
Forkhead box C1 (FOXC1), 1293
Forkhead Box F1 (FOXF1), 1259
Formagraft, 1233–1234
Forticell Biosciences, 1337
Fostering regeneration, 150–151
4D bioprinting, 1400
Foxd1, 811–812
Foxd1 deletion, 812
FOXF1, 1262
FOXM1, 1262–1263
Foxn1, 692–695
FOXO1, 1262
Fracture healing, 919–922
Fracture-induced bone, cartilage, stromal progenitor (f-BCSP), 1011–1012
Fragile X syndrome, 448–449
Free-edge effect, 1312
Free fatty acids (FFAs), 1360
Free-form fabrication technologies, 1275–1276
Freeze-drying process, 1066, 1073, 1235
FTOC. *See* Fetal thymic organ culture
Fuchs' endothelial corneal dystrophy, 1139
Full circumferential repair, 853*t*, 854
Full-thickness chondral defects, 1499–1500
Full-thickness skin grafts, 1322
Full thickness wounds, 1321–1322
Functional electrical stimulation, 1040
Function-blocking antibodies, 99
Fuscinosis, neuronal lipoid, 7
Fused deposition modeling (FDM), 304
Fusogen, 1076, 1078*f*
Fusogenic peptides, 535

G

GABAergic cells, 1031–1032
G-actin, 69–70
GAGs. *See* Glycosaminoglycans
GALA, 535
Galardin, 1314
Galectin-3, 836*t*
γ-interferon, 1493–1494
Gastrointestinal tract organoid, 1541–1542, 1542*f*
Gastroschisis (GS), 461
Gastrulation, 723–724, 806
Gata3, 807
Gaze-contingent remapping, 1166–1167
G-code, 996
Gdf11 gene transfer, 1196
Gdnf expression, 808–809
Gelatin, 320, 564, 831*t*, 1208–1209, 1398, 1404*t*
Gelatinase A (MMP-2), 1314
Gelatin-based hydrogel materials, 896
Gelatin methacrylate (GelMA), 898, 982, 983*f*, 984–985, 997–998, 1395–1398, 1400, 1423*t*, 1430–1434
Gellan gum, 324
Gels, 334
  biologic, 625
Gem-21, 1228
Gene-activated matrix, 1231–1232
Gene activity, controls of, 67–68
Gene delivery, 523, 540
  approaches, 520*f*
  engineering tissue with, 538
  fundamentals of, 519–521
  material-mediated, 538
  multiple, 538
  nonviral vehicles for, 533*f*
  plasmid, 525–526
  and tissue engineering, 542*f*
  vehicles, 521*f*
Gene editing, 453, 520, 779, 1587
  gene transfer vectors for, 435, 507–508
Gene expression, 40, 519, 530
  control of, 66–67
  gene transfer to regulate, 509
  inducible, 505
  regulation of, 505
Gene gun, 954–955
Genes
  proneural, 147
  protein coding, 66
  replacement or addition, 519
  structure, 67*f*
Gene therapist, 493–494
Gene therapy, 493, 519, 629–630, 947, 1029, 1095, 1100, 1172–1175, 1195–1196
  adeno-associated virus (AAV)-based, 493
  barriers, 521
  challenges to, 509–510
  critical decisions in strategizing, 495
  cultured epidermal autografts with, 1302
  ex vivo *vs.* in vivo, 494
  intervertebral disk (IVD) regeneration, 953–955
    endogenous gene regulation, 955
    nonviral, 954–955
    viral, 954
  mesenchymal stem cells (MSCs), 901
  with nuclear transfer and stem-cell therapy, 509*f*
  for osteoarthrosis, 541
  for rheumatoid arthritis, 541
  strategies for, 493–495, 494*f*
  for transdifferentiation, 1099–1101
  in utero, risks of, 385
  viral, 526–527
Gene therapy vectors, 496*f*
  cell receptors of commonly used viral-based, 498*t*
  genetic modification of, 502–505
Genetic hearing loss, 1093
Genetic lineage tracing, 1194

Genetic medicine, for tissue engineering, 541
Genetic modification, 1093
Genetic reprogramming, 471–472
Genetic techniques, 471–472
Gene transfection, 1211
Gene transfer, 377
  application, 493, 497
  to control uncontrolled stem-cell growth, 508
  genetic deafness, 1099
  to instruct stem-cell differentiation, 508–509
  issues relating to successful, 494*f*
  to regulate gene expression, 509
  to stem cells, 508
  with stem-cell strategies, 508–509
  strategies, 508–509
  vectors, 496*f*, 496*t*
  , characteristics
Gene transfer vectors, 495–502
  for gene editing, 507–508
  modification of, 502
  tropism of, 504*f*
Genetically engineered insulin, 1590
Genipin cross-linked fibrin hydrogels, 943–944
Genome-wide association studies (GWAS), 450
Genome-wide libraries, 538
Genomic integration, associated insertional mutagenesis, 385–386
Genomic stability, 448
Gentamicin, 1094–1095
Gentamicin-induced hair cell apoptotic processes, 1095–1096
Geographic atrophy (GA), 1147
Geranylgeranylacetone, 1098
Gierer–Meinhardt (GM) model, 44
GINTUIT, 54, 1337, 1341
Glaucoma, 1145, 1147, 1148*t*, 1153–1154
  cell-based therapies, 1148*t*
  stem cell–based therapies to, 1154–1155
Glaucomatous optic neuropathy (GON), 1153–1154
Glial cell, 1425
Glial-line-derived neurotrophic factor (GDNF), 222–223, 808–809, 1028–1029, 1031, 1051, 1098
Glioblastoma-associated macrophages, 1428
Glioblastoma multiforme, 1428
Glioblastoma tumor, 1428
Glioma-associated oncogene 1 (Gli1), 1295
Glioma tumors, 1029
Gliosis, 1050–1051
Globin-switching, 767–768
Glucocorticoids, 1155
Glucose, 1461–1462, 1464, 1464*f*
Glucose-metabolism, 665–666
Glucose-responsive stem cell, 666–667
Glutamate, 1146–1147
Glutathione, 1094–1096
Glycidyl methacrylate hyaluronic acid (GMHA), 1430–1431
Glycol, monomethoxy poly(ethylene), 505
Glycolic acid, 318
Glycolysis, 68
  inhibition of, 712
Glycopolymers, synthetic cationic, 536
Glycoproteins
  adhesive, 123–127
  extracellular matrix (ECM), 135–136
  WNT, 694
Glycosaminoglycans (GAGs), 72, 127, 135–136, 159–160, 229, 322, 324–325, 345, 635–636, 760, 865, 940–942, 944, 1059, 1067–1069, 1212–1214, 1398, 1406, 1502
GMP. *See* Good manufacturing practice
GMP/GTPs. *See* Good manufacturing practices/good tissue practices
Goblet cell and squamous metaplasia, 1257
Goblet cells, 709–710
Good Laboratory Practices (GLP), FDA, 55
Good manufacturing practice (GMP), 56–57, 59–60, 191
Good tissue practices (GTPs), 56–59, 1475*t*
Gore-Tex patches, 459, 623*f*
Governmental payers, reimbursement, 1580
G-protein-coupled receptor 44 (GPR44), 1299–1300
G-protein-linked receptors, 73–74
Gradual distraction, 1008
Graft failure, 157
Graft-induced dyskinesias, 1026
Graft infection, 628
Graft outcome, cellular and molecular mediators of
  biological modification, 628
  cell therapy, 630
  cellular recruitment, 626
  compliance, 628
  gene therapy, 629–630
  physical/chemical modification, 626–630
  porosity, 627–628
  protein adsorption, 627
  protein therapy, 629
  resistance to infection, 628
  surface characteristics, 626
  surface modifications, 626–627
  thromboresistance, 627
Grafts in clinical use
  cryovessels, 622
  Dacron, 622
  synthetic grafts, 622–623
Grafts in patients
  conduit patency and failure, 618–619
  hemodialysis vascular access, 619–620
  host environment, 621
  inflammation and host response, 620–621
  venous reconstruction, 619
Graft-*versus*-host-disease, 891, 1007
Granulation tissue, 1312–1316, 1313*f*
  fibroplasia, 1313–1315
  neovascularization, 1315–1316
Granulocyte-colony stimulating factor (G-CSF), 757–758, 778, 835–836, 836*t*
Granulocyte–macrophage-colony stimulating factor (GM-CSF), 757–758
  receptors, mutation in, 1266
Granulocytes, neutrophils, 778–779
Granulosa cell spheroids, 866
Gravity-driven perfusion, 1278–1279
Green fluorescent protein (GFP), 1225–1226, 1294
  -labeled MSCs, 864
Green fluorescent protein-tagged H2B histone (H2B-GFP) pulse-chase system, 1290–1291
Greenhouse gas (GHG) emissions, 1356, 1369
Griffonia simplicifolia lectin, 1278
gRNA. *See* Guide RNA
Growth and death, 74
Growth and differentiation factor-5 (GDF-5), 948, 949*t*, 952
Growth and differentiation factor-6 (GDF-6), 949*t*, 952
Growth factor receptors, regulation of, 94
Growth factors, 758–759, 765, 850–851, 888, 894, 896–897, 949*t*, 971, 980, 991–992, 995, 1011–1012, 1028, 1192–1193, 1195–1196, 1209, 1213–1214, 1310, 1312–1314, 1316, 1324, 1377–1378
  articular cartilage injury, 973–974
  biomimetic-driven strategies, 923–924, 923*t*, 924*t*, 925*t*
  cartilage tissue for craniofacial complex, 1213
  drawback to, 1210
  imbalances, 1334
  intervertebral disk (IVD) regeneration, 948–953
    in vitro studies, 948–952, 949*t*
    in vivo studies, 952–953
  positive biochemical stimulation, 1213
Growth medium cocktails, 886
GS. *See* Gastroschisis
GTPases, 70–71
GTPs. *See* Good tissue practices
Guanine nucleotide exchange factor (GEF), 105–107
Guanosine diphosphate (GDP), 69, 1575
Guanosine triphosphate (GTP), 69
Guided tissue/bone regeneration (GTR/GBR), 1221
Guide RNA (gRNA), 520

H

HA-coated PLGA scaffolds, 1015
hADSC-derived osteogenic cells, 926
hAECs. *See* Human amnion epithelial cells
Hair cells, 1100
  interventions for, 1099–1101
  replacement, 1101
Hair dermal papilla cells, 1191
Hair follicles (HFs), 1289, 1332
  anatomy, 1291, 1292*f*
  cycle, 1291, 1292*f*
  and immune system, 1300–1301
Hair follicle stem cells (HFSCs), 1291–1295
  bulge as stem cell source, 1291–1292, 1292*f*
    characteristics of, 1292–1294
    molecular signature, 1293–1294, 1293*t*
    multipotency, 1294
    quiescence, 1292–1293

ectodermal appendages, 1295–1297
nail, 1296–1297
sebaceous glands (SGs), 1295–1296
sweat glands, 1296
marker expression, subpopulations by, 1294–1295
bulge, 1294–1295
upper hair follicle, 1295
skin homeostasis, 1297
wound healing, 1297–1298
wound-induced hair follicle neogenesis and regeneration, 1298
Hair germ (HG), 1293*t*
Hair primordia-bearing organoids, 1301
Hairy-cell leukemia, T cell lymphocytes, 1588–1589
Halorhodopsin, 1174–1175
Hamburger cultured from bovine myoblasts, 1359*f*
Haptoglobin, 788
Hard scaffold, 1240*t*
HARV. *See* High-aspect rotating vessel
Hassall's corpuscles, 683
Haversian systems, 919–922, 929
Hayflick limit, 1358
HB. *See* Hemophilia B
Hb-based O_2 carriers (HBOCs), 785, 787–792, 794
Hb extravasation, 788*f*
HBMSC. *See* Human bone marrow stromal cell
Hb polymerization, 789–790
Hb toxicity, 788
HCC. *See* Hepatocellular carcinoma
HCT-116 colon carcinoma cells, 1448–1449
HDAC4. *See* Histone deacetylase 4
Health Canada, 1568
Healthcare Common Procedure Coding System (HCPCS), 1581
Healthy life expectancy (HALE), 1575
Hearing, 238
Hearing, protection and repair of
acoustic trauma, prevention of, 1096–1097
acquired sensory hair cell loss, protection from, 1093–1094
antiinflammatory agents, 1097
auditory nerve replacement, 1101
calcium influx, 1094, 1097
endoplasmic reticulum stress, 1094
excitotoxicity, protection from, 1098–1099
gene transfer, genetic deafness, 1099
hair cell repair, interventions for, 1099–1101
hair cell replacement, 1101
heat shock proteins (HSP), 1097–1098
neurotrophic factors, 1098–1099
ototoxicity, prevention of, 1094–1096
oxidative stress, 1094
repair/replacement, interventions for
central auditory prostheses, 1102–1103
cochlear fluids, local delivery to, 1103
cochlear prostheses, 1101
fully implantable cochlear prostheses, 1101–1102, 1102*f*
stress-related mitochondrial pathways, 1094
Heart, electrical coupling of cardiomyocytes on, 608–609
Heart failure, 594
Heart-on-a-chip, 1448
Heart tissue models, 1428–1430, 1429*f*
Heart valves, 641*f*
biocompatibility, 638–639, 642
biomaterials and scaffolds, 640–643
cell seeding techniques, 644–645
cell sources, 643–644
cellular biology of, 636–637
innervation and vasculature, 637
valvular endothelial cells (VECs), 636
valvular interstitial cells (VICs), 636–638
dysfunction, 637–638
function and structure, 635–636
prosthetic, 638
replacement, 638–648
semilunar, 635–636
tissue engineered, 636
valvular repair and remodeling, 638
Heat shock factor 1 (HSF1), 1098
Heat shock proteins (HSP), 1097–1098
Hedgehog (Hh) pathway, 85, 714, 809, 1541
HeLa cancer cells, 1401–1403
HeLa cells, 1427–1428
Helicobacter pylori infection, 1541–1542
Helix-loop-helix (HLH)
family proteins, 152
transcription factors, 147
Helix pomatia lectin, 1278
Hemangioblasts, 769–770
Hematopoiesis, 765–767
Hematopoietic hierarchy, 766*f*
Hematopoietic malignancies, 873
Hematopoietic progenitor cells (HPCs), 684
Hematopoietic stem cells (HSCs), 370–371, 375, 684, 757, 758*f*, 759*t*, 765, 846, 1029, 1124, 1155
applications, 757–758
multilineage differentiation, manipulation of, 760
for in vitro expansion, 759–760, 760*f*
in vivo tracking hematopoietic stem cells, 761
autologous, 527
biomaterials, effects of, 758–759, 759*t*
biomimicking materials, 759, 761
$CD34^+$ cells, 1124, 1125*f*
lentivirus-transduced autologous, 502
niche, 757–760, 760*f*
postnatal HSC transplantation, 371
Hematopoietic stem cell transplantations (HSCTs), 765
Hematopoietic stem/progenitor cells (HSPCs), 684–685
Hematopoietic/vascular progenitors (CD34/endothelial colony-forming cells), 1123–1124
Hemicystoplasty, 853*t*
Hemidesmosome component collagen XVII (COL17A1), 1291
Hemidesmosomes, 240–241
Hemi-mandible, regeneration of, 1210*f*
Hemodialysis vascular access, 619–620
Hemodynamic forces, 636
Hemoglobin, sources of, 791–792
Hemoglobin toxicity, 787–788
Hemolink, 790
Hemopexin, 788
Hemophilia A (HA), 377–378
feasibility and justification for treating, 380–382
preclinical animal models for, 378–379
preclinical model of, sheep as, 379–380
prior to birth, 380–382
therapeutics, 383–385
treatment, 378–382, 384–385
Hemophilia B (HB), 378
Hemopure, 790, 794–795
Hemorrhagic anemia, 767
Hemosol Inc, 790
HEMOXYCarrier, 793
Heparan sulfate (HS) proteoglycan, 227–228
Heparan sulfate proteoglycans (HSPGs), 94, 96–97, 100–101
Heparin, 627, 1316
Heparin binding delivery system, fibrin scaffold and, 1072*f*
Heparin-binding epidermal growth factor (HB-EGF), 1312
Hepatic cells, 729*f*
Hepatic function, 723
Hepatic stem cells (HpSC), 7–8, 724
Hepatic tissue engineering, 741*f*
Hepatoblast, 726–727
differentiation, 723–724
Hepatocellular carcinoma (HCC), 730, 1431–1432
Hepatocyte, transplantation, 730
Hepatocyte-derived organoids, 728–729
Hepatocyte growth factor (HGF), 84, 224, 836*t*
Hepatocyte-like cells, 883
Hepatocytes, 723, 725–727, 744, 1377–1378, 1417
hybrid, 725
to liver progenitors, 730–731
in organ regeneration, 724–726
reprogrammed, 744
role in liver regeneration, 725
transplanted, 743
vs. biliary epithelial cells (BECs), 723–724
Hepatocyte transplantation (HT), 740
Hepatotoxicity, 438
HepG2 hepatoma cells, 1447–1449
Hereditary disorder, 495
Herpes simplex virus type-1 (HSV-1), 528
hESCs. *See* Human embryonic stem cells
Hesticular cells (HTO), 873, 874*f*, 875*f*
Heterodimers, integrins, 94
Heterogeneity, 889, 1011, 1017
Heterogeneous osteosarcoma cells, 1016
Heterotopic ossification, 930
Heterotopic transplantation, 1030–1031
Hexon, 497
hFLMPCs, 724
HGF. *See* Hepatocyte growth factor
HiDep-1, 767–768

High-aspect rotating vessel (HARV), 189−190, 471
High hydrostatic pressure (HHP), 864
High-level illumination photoreceptor injury model, 1150−1151
High-risk devices, 1563
High-throughput screening, 537−538
High-yield photoconversion systems, 1174
HIO. *See* Human intestinal organoids
Hippo signaling pathway, 244−245
Histocompatability antigens (HLA), 1493−1494
Histomorphometric analysis, 1010
Histone deacetylase 4 (HDAC4), 149
Histone deacetylase (HDAC) inhibitor, 836*t*, 1095
Hnf1b, 811
Hodgkin's lymphoma, 872−873
Hoescht 33342, 1118
Hollow fibers, 286
Holoclar, 1138
Homeobox (Hox) family, 757
Homeostasis, 133, 887, 1289−1290, 1332, 1443
  intestinal, 710
  tissue development and, 119
Homograft, cryopreserved, 639−640
Homopolymers, 282
  surface adsorption of, 284
Host defense, immune cell functions beyond, 365
Host immune system, 403−404, 405*f*
Host immunobiology, MSC, 889−891
Host response, 363−364, 366
Hoxa3 mutants, 691
HoxB4, 807
Hoxd11 transgene, 807
Hox genes, 807−808
HPG. *See* Hyperbranched polyglycerol
HpSC. *See* Hepatic stem cells
HSC. *See* Hematopoietic stem cells
HSPGs. *See* Heparan sulfate proteoglycans
HSV-1. *See* Herpes simplex virus type-1
HT. *See* Hepatocyte transplantation
H-type vessels, 265−266
HuCNS-SC, 7
Humacyte, 624
Human amnion epithelial cells (hAECs), 458
  allogeneic, 458
Human amniotic fluid stem cells (hAFSCs), 829
Human articular cartilage, 887*f*
Human blastocysts, inner cell mass (ICM) of, 467
Human bone marrow-derived mesenchymal stem cells (hBMSCs), 561
Human bone marrow stromal cell (HBMSC), 40
Human breast cells, 98
Human cardiomyocytes, 1448
Human cells, tissues, and cellular and tissue-based products (HCT/Ps), 1561−1562, 1565−1566
  homologous use, 1566
  minimal manipulation, 1565−1566
Human cell, tissue, and cellular and tissue-based product (HCT/P), 58−60
Human cervical cancer HeLa, 1424
Human chorionic gonadotropin (hCG), 873−874
Human CLiPs (hCLiPs), 730−731
Human cord blood-derived EPCs, 926
Human corporal smooth muscle cells (SMC), 875−876
Human-derived serum, 887
Human dermal fibroblasts, 447*f*
Human *EHMT2* gene, 1097
Human embryonic kidney cells (HEK293), 406−407
Human embryonic stem cells (hESCs), 188, 421, 438, 445, 452, 457, 560, 598, 767, 768*f*, 772, 828, 1149, 1151, 1156, 1578
  approaches to, 421−425
  clinical application of, 457, 480−482
  cultivation of, 470−471
  culture of, 426, 475
  derivation of, 421, 426−428, 469, 482
  differentiation of, 473−474, 476, 479
  directed differentiation, 426−430
  early stages of, 423*f*
  efficient transfection of, 472
  erythroid cells, large-scale production of, 768*f*
  maintenance of, 425
  MKs and platelets derived from, 769*f*
  morphology and markers of, 422*f*
  naive, 424
  production of, 470
  safety concerns, 430−431
  self-renewal of, 469−470
  spontaneous differentiation of, 427*f*
  subculture of, 425−426
  undifferentiated, 479
  vascular differentiation of, 477−478
  in vivo differentiation of, 472−474, 657
Human embryonic stem cells-derived hemangioblasts, 1156*f*
Human embryonic stem cells-derived photoreceptor progenitors engraft, 1152*f*
Human ether-a-go-go-related gene (hERG), 437−438
Human gingiva, 1224
Human HF stem cells, markers of, 1293*t*
Human induced pluripotent stem cells (hiPSCs), 598, 830, 1013, 1138−1139, 1149, 1151, 1154, 1156, 1524, 1529
  hiPSCs-derived cardiomyocytes, 1429, 1529
  hiPSCs−derived mini-brain model, 1425−1426
Human intestinal organoids (HIO), 714−715, 717
Humanitarian device exemption (HDE), 1493
Humanitarian Use Devices, 1563
Human keratolimbal autografts, 1118
Human leukocyte antigen (HLA), 451, 765
Human limbal autografts, 1118
Human liver carcinoma cell line HepG2, 1424
Human mesenchymal stem cells (hMSCs), 301, 887−888, 984−985, 1395
Human muscle progenitor cell (hMPC)−laden hydrogel bioink, 1407
Human nonsmall cell lung cancer, 1449
Human patellofemoral articular cartilage, 969
Human pluripotent stem cells (hPSCs), 815, 817
Human progenitors, markers of, 1293*t*
Human rotator cuff−derived cell sheets, 994
Human retinal progenitor cells (hRPCs), 1152−1153
Human serum albumin (HSA), 790−791
Human skin constructs (HSCs), 1301−1302
Human skin equivalents (HSEs), 1331, 1337−1340
Human skin fibroblasts (HSFs), 1432−1434
Human skull, bones, 1202*f*
Human umbilical cord blood−derived MSCs (hUCB-MSCs), 1505
Human umbilical vein endothelial cells (HUVEC), 97, 1278−1279, 1395, 1398, 1400, 1401*f*, 1408, 1430−1435
Humoral (antibody-mediated) immune responses, 1338
Huntington's disease (HD), 1025, 1031
  cell replacement strategies, 1031
HUVEC. *See* Human umbilical vein endothelial cells
Hyaline cartilage, 940, 942−943, 971, 979, 985
Hyaline cartilage endplates, 940
Hyalofast, 1503
Hyaluronan, 95, 127−128, 127*f*, 324−325, 896, 1323, 1502
  Synthesis, inhibition of, 96−97
Hyaluronan benzyl ester (HYAFF 11) scaffolds, 562
Hyaluronan-binding proteoglycans, 127−128
Hyaluronate, 929
Hyaluronic acid (HA), 139, 562, 899, 1053*t*, 1059−1065, 1066*f*, 1067*f*, 1208−1209, 1398, 1409, 1423*t*
Hyaluronic acid and methylcellulose (HAMC), 1059
Hybprinter, 1396
Hybrid 3D bioprinting technology, 1396
Hybrid bioinks, 13
Hybrid brain−machine interfaces (BMIs), 1042
Hybrid electroacoustic stimulator, 1102
Hybrid electrodes, 1101
Hybrid fabrication system, 1396
Hybrid hepatocytes, 725
Hybrid hydrogels, 1015
Hybrid polymers, 981−982
  with immobilized functional groups, 283−284
Hybrid scaffolds, 899−900, 981−982
Hybrid surgical approaches, 1209−1210, 1211*f*
Hydraulic elevation, 261−262
Hydrogel-based bioinks for cell-based bioprinting, 1396−1399
  naturally derived hydrogels, 1398−1399
  required properties of, 1396−1397, 1397*f*
  synthetic hydrogels, 1397−1398

tissue-specific extracellular matrix–based hydrogels, 1399
Hydrogel-based systems, 866
Hydrogel fabrication, with microfluidics, 194
Hydrogels, 2–4, 13–14, 475, 562–563, 758–760, 895, 921*t*, 926, 982–983, 993, 1015, 1058, 1072–1073, 1233, 1235–1236, 1323, 1524
cylindrical tough mesh coated with, 998*f*
fiber-reinforced, 162
macroporous, 287
multidomain peptides (MDPs), 4
natural polymer, 301
Hydrogel scaffold, 996*f*, 1501
Hydrogen peroxide (H_2O_2), 787–788, 972
Hydrophilic scaffolds, 352
Hydrophilic synthetic polymers, 1212
Hydrophobic polymer poly(propylene fumarate), 1212
Hydrostatic pressure, 895
Hydroxyapatite (HAP)–tricalcium phosphate (TCP), 889
Hydroxylation, proline, 121
Hyperbranched polyglycerol (HPG), 284
Hyperglycemia, 1155
Hyperplasia, myointimal, 618–619, 623
Hypertension, pulmonary, 209–210
Hypertrophic nonunion, 929–930
Hypertrophic scars, 1318–1319
cellular and extracellular matrix characteristics of, 1319*t*
Hypertrophy, chronic hypertension induced left ventricle, 611
Hypertropic nonunion, 929–930
Hypogonadal disorders, 873–874
Hypothalamic–pituitary–ovarian axis, 865–866
Hypoxia, 167, 895, 1096, 1124, 1333–1335
Hypoxia inducible factor-1α (HIF-1α), 1315
Hypoxia-inducible transcription factors, 1334–1335
Hypoxic expansion of stem cells, 947
Hypoxic gradient, 1542–1543
Hysterectomy, 863

I

IBMX, 1360
iCasp9, 771–772
ICM. *See* Inner cell mass
Idiopathic pulmonary fibrosis, 1274
Idiopathic pulmonary hypertension, 1260
IkB kinase complex-associated protein *(IKBKAP)*, 437
Ilizarov devices, distraction osteogenesis with, 1511
ILK. *See* Integrin-linked kinase
Imaging technologies, 16
Imiquimod, 1301
Immature chondrocytes, 967
Immobilized functional groups, hybrid polymers with, 283–284
Immobilizing peptide ligands, 350–351
Immortal human neural stem cells, 1028
Immortalization techniques, 1538
Immortalized cell lines, 406–407
Immune cell behaviour, in transplant niche, 412
Immune cells, 1273
roles in building tissues after injury, 363–365
targeting tissue engineering/regenerative medicine therapies, 366
Immune responses, 84
to implanted materials, 410–412
Immune system, 137
components of, 1486
dendritic cells of, 1332
hair follicles, 1300–1301
host, 403–404, 405*f*
Immunoglobulin, 72
Immunoisolation, 482
barrier, 404–405, 406*f*, 409
devices, 403–405, 404*f*, 406*f*, 408–410, 409*f*, 412
Immunology, prenatal therapy, 460
Immunomodulation, 364, 891, 1051–1052
Immunomodulatory cytokines, 849–850
Immunomodulatory materials, 672
Immunoprotected cells, oxygenation of, 409–410
Immunoselection, magnetic, 479–480
Immunosuppressive drugs, 421, 481, 1155
Immunotherapy, tissue engineering/regenerative medicine strategies as, 365–366
Impaired healing and mechanisms, 1333–1335
abnormalities at cellular level, 1335
acute *vs.* chronic wound healing, 1333
bacterial colonization, 1333–1334
growth factor imbalances, 1334
ischemia, 1334–1335
matrix metalloproteinase activity, 1334
moist wound healing in chronic wounds, 1334
Impaired vision, 1163
iMPCs, 151
Implantation models, 927
Implanted electrode arrays, 1174
Implanted materials, immune responses to, 410–412
Implants, synthetic, 559
Inborn liver-based errors of metabolism, 737
Indirect delivery methods, 1211
Indirect ex vivo approach, 1211
Indirect pathway of transplant rejection, 1493–1494
Indoleamine 2,3-dioxygenase (IDO), 890
Induced pluripotent stem cells (iPSCs), 5–7, 23, 37, 111, 150–151, 184, 421, 435–436, 445, 458, 467–469, 560, 594, 741, 767–768, 773, 777–778, 815, 826, 846, 1010, 1012–1013, 1027–1028, 1115, 1123, 1137, 1171, 1276–1277, 1424–1425, 1454–1455, 1579–1580
applications of, 448, 449*f*, 452
autologous transplantation of, 451
cellular products, 439*t*
iPSC–derived oligodendrocytes, 1059
iPSC-derived photoreceptors, 1123
disease-modifying potential of, 451–452
genomic variation in, 448
isogenic control, 435
kidney, tissue engineering of, 830
product development, business issues, 1579–1580
production of, 445, 447
pulmonary disease, treatment of, 1263–1264, 1264*f*
pulmonary endothelial engineering, 1278
to pulmonary epithelial cell lineages, 1264–1265
relative immaturity of, 435–436
in retinal regeneration, 1121, 1122*f*
hematopoietic/vascular progenitors (CD34/endothelial colony-forming cells), 1123–1124
photoreceptors from, 1122–1123
retinal ganglion cells from, 1123
retinal pigment epithelial from, 1121–1122
therapeutic potential of, 458
Industrial bioreactors, 1379–1380
Industrial production, cultured meat technology, 1375–1382
associated dangers and risks, 1381
atrophy and exercise, 1380
consumer acceptance and perception, 1382, 1383*t*
fields, 1380
industrial bioreactors, 1379–1380
meat processing technology, 1381
muscle fibers, tissue engineering of, 1378
regulatory issues, 1381–1382
safe differentiation media, muscle cells, 1377–1378
safe media for stem cells, 1377
scaffolds, 1378–1379
senescence, 1381
stem cell lines from farm-animal species, 1376–1377
Industry-driven consortium effort, 1472
Indwelling urethral catheterization, 855
Infertility, 871
Inflammation, 287–288, 1310
chronic, 111
and host response to interventions and grafts, 620–621
neutrophils, 1310
platelet, 1310
transition to repair, 1310
wound repair, 1310
Inflammatory memory, 1301
Inflammatory memory of skin cells, 1301
Inflammatory response, to vascular interventions, 620
Informed consent, 1590
INFUSE, 930
Infuse Bone Graft, 930, 1016, 1233–1234, 1512–1514
Initial Targeted Engagement for Regulatory Advice on CBER products (INTERACT) program, 1557
Injectable biomaterials, 2–4
Injectable carrier/soft scaffold, 1240*t*

Injectable scaffolds, 563
Injury-regeneration balance, 1192–1193
Inkjet printing, 12, 301, 303–304, 925*t*, 982–983, 1396, 1403–1406, 1408–1409, 1418–1420, 1419*t*
  electromagnetic inkjet printing, 1419
  low-viscosity materials, 1418–1420
  piezoelectric inkjet printing, 1418–1419
  thermal inject printing, 1418
  water-based materials, 1418–1420
Inlay.onlay repair, 853*t*
Inlay procedures, 854
Inner cell mass (ICM), 421–422, 425
  of human blastocysts, 467
Inner hair cells (IHCs), 1094, 1097–1099
Inner limiting membrane (ILM), 1146–1147
Inner nuclear layer (INL), 1146–1147, 1174
Inner plexiform layer (IPL), 1146–1147
Innervation, in bioengineered tissue, 2
Inorganic nanoparticles, 537
Insect ecdysone receptor system, 507
Insertional mutagenesis, 495, 502, 1012–1013
  genomic integration associated, 385–386
In silico breast cancer models, 570–571
In situ polymerization, 1241
In situ recellularization strategies, 1527
Institutional review boards (IRB) review, 1557
Insulin, 403, 1360
  producing cell sources, 667*f*
Insulin growth factor (IGF) signaling, 85, 1209, 1312
Insulin-like growth factor-1 (IGF-1), 164, 888, 948, 949*t*, 1009, 1014
Insulin-like growth factor-2 (IGF-2), 1014, 1377–1378
Insulin-producing β cells, 474
Integrase-deficient lentiviral vectors, 528
Integrated multiorganoid model systems, 1449–1455, 1450*f*
  additional disease modeling, 1452
  cancer, 1450
  drug testing/toxicology, 1450–1451, 1451*f*, 1452*f*
Integrated tissue-organ printer (ITOP), 12, 300
Integrin-binding motifs, 993
Integrin-linked kinase (ILK), 84, 109–110
Integrins, 72, 107, 228, 1311, 1313*f*, 1316
  cytoplasmic domain of, 80–81
  heterodimers, 94
  ligation, 110
  misexpression of, 80–81
  presence and function of, 81
  receptors, 529
  superfamily, 1311, 1311*t*
Intellectual property (IP), 55
Intelligent scaffolds, 1323
Interferon-γ (IFN-γ), 774–776, 889, 919
Interferon regulatory factor 6 (IRF6), 1296
Interfollicular epidermal stem cells, 1289–1291
  epidermal proliferative unit *vs.* committed progenitor, 1290–1291
  label-retaining cell (LRC) methods, 1291–1292
Interfollicular epidermis (IFE), 1289
Interfollicular stem cells, 1310–1311
Interleukin-1 (IL-1), 164–165
Interleukin-3 (IL-3), 767, 1310
Interleukin-6 (IL-6), 849–850, 888
Interleukin (IL)-8, 849–850
Interleukin (IL)-10, 890, 919
Intermediate filaments, 239
Intermediate macrophages, 1310
Intermediate mesoderm (IM), 806–809
International Cartilage Repair Society, 985
International Council of Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), 1568
International Organization for Standardization (ISO), 1474
International Pharmaceutical Regulators Program (IPRP) Cell Therapy Working Group (CTWG), 1568
International Society of Cell Therapy, 884–885, 917–918, 1119
Interstitial collagenase (MMP-1), 1317, 1334
Interstitial lung diseases (ILD), 1253
  pulmonary macrophage transplantation, 1266
Interventricular septum (IVS), 579
Intervertebral disk (IVD), 937, 941*t*
  cell-biomaterial constructs, 940–945
    annulus fibrosus repair and regeneration, 942–944, 942*f*, 943*f*
    composite cell-biomaterial intervertebral disk implants, 944–945, 945*f*, 946*f*
    nucleus pulposus cell-biomaterial implants, 940–942
  cell therapy
    clinical studies, 947–948
    preclinical studies, 946–947
  cellular engineering, 945–948
  composition and mechanical properties, 939*t*
  gene therapy for, 953–955
    endogenous gene regulation, 955
    nonviral, 954–955
    viral, 954
  growth factors, 948–953
    in vitro studies, 948–952, 949*t*
    in vivo studies, 952–953
  spinal motion segment, 937*f*
  structure and function, 938–940, 938*f*
  in vivo preclinical models for, 955–957
  in vivo studies, other biologics, 953
Intestinal epithelial cells, 709–710
Intestinal epithelial layer, 709–710
Intestinal epithelium, 709
  signaling pathways in, 712
Intestinal homeostasis, 710
Intestinal organoids, 717, 1543
Intestinal progenitor cells, 710
Intestinal stem cells (ISCs), 709–710, 712
  asymmetrical division of, 712
Intestinal submucosa, 940–942
Intestine, stem cells of, 709–714
Intraarticular fractures, 972
Intracellular proteins, 69
Intracellular trafficking, 523–526
Intracochlear pressure sensor, prototype of, 1102*f*
Intracochlear transducer, 1101–1102
Intracutaneous transplantation of bone marrow–derived macrophages, 1300–1301
Intracytoplasmic sperm injection (ICSI), 871
Intralaminar fenestration surgical technique, 1214
Intramembranous and endochondral ossification, 919–921
Intraocular pressure (IOP), 1145, 1153
Intraperitoneal space, 409
Intrathecal hyaluronic acid and methylcellulose system, 1059
In utero gene therapy (IUGT), 369–370, 377–378, 387
  rationale for, 370–371, 376–378
  risks of, 385
In utero transplantation (IUTx), 369–376, 387
  barriers to, 373–376
  clinical experience with, 376
  experiments in large preclinical animal models, 372–373
  murine experiments with, 372
Invariant NKT (iNKT), 775–776
Investigational device exemption (IDE) application, 1473, 1563
Investigational new drug (IND) application, 1472, 1476, 1557–1559
  charging for investigational drugs, 1566
  chemistry, manufacturing, and controls information, 1558
  clinical protocol, 1559
  cross-reference authorization, 1559
  original investigational new drug application submission, 1559
  pharmacology and toxicology data, 1558
  previous human experience, 1558
  required US FDA forms, 1557–1558
Investigational new drug application/biologics licensing application (IND/BLA) pathway, 1553
Investigator's Brochure (IB), 1558
In viral technologies, 520–521
In vitro cell culture methods, 275–278
  adhesion and spreading, 275–277
  aggregation, 278
  cell phenotype, 278
  migration, 277–278
In vitro cell cycle studies, 887
In vitro chondrogenesis, 1462
In vitro culture parameters, engineered tissues, 161–171
  biomaterials, 162–165
    cardiac TE, 165
    cartilage TE, 162–165
  bioreactors and growth factors, 166–169
    cardiac tissue-engineering bioreactors, 168–169, 169*f*
    cartilage tissue-engineering bioreactors, 167–168, 168*f*
    cell seeding, 166–167
    construct cultivation, 167

bioreactors and mechanical forces, 169–171
cartilage tissue, mechanical effects on engineered, 170–171
electromechanical effects on engineered myocardium, 171
hydrodynamic forces effects, 169–170
mechanical tension, compression, and shear loading, 170
culture duration, 161–162
cardiac TE, 162
cartilage TE, 161–162, 161*f*
In vitro differentiation protocols, for human embryonic stem cells, 657
In vitro fertilization (IVF), 863, 873
In vitro-grown tissue-engineered constructs, 159
In vitro meat production system, 1371
In vitro studies, 160, 740–743, 926–927, 1400–1403, 1402*t*
culture conditions, 158–159
for engineering functional tissues, 159–161
intervertebral disk (IVD) regeneration growth factors (GFs), 948–952, 949*t*
monolayer tissue growth, 42
three-dimensional tissue growth, 43–44
tissue growth on complex surfaces, 42–43
tissue-specific models, 1403
tumor models, 1401–1403
two-dimensional (2D) culture models, 179
In vitro T cell differentiation, 683–685
In vitro tissue-engineered vascular grafts, 623–625
In vitro trial, 436–437
In vivo bioreactor (IVB), 261, 262*f*, 1209–1210
cartilage engineering in, 265*f*
conceptual framework of, 261
creation of, 262*f*
formation of cartilage in, 264*f*
In vivo bone engineering, 261–264
In vivo cartilage engineering, 264–265
In vivo fate mapping of cardiac progenitors, 582
In vivo gene therapy, 495, 901
vs. ex vivo gene therapy, 494
In vivo homologous recombination, 1210–1211
In vivo recellularization of decellularized scaffold, 1525–1526
In vivo setting, cell interactions unique to, 287–289
In vivo spinal fusion models, 927
In vivo studies, 159, 278–280, 743, 929
for craniofacial bone, 1208–1209
intervertebral disk (IVD) regeneration growth factors (GFs), 952–953
preclinical models for, 955–957
intervertebral disk (IVD) regeneration, other biologics, 953
In vivo tissue-engineered vascular grafts, 625
In vivo tracking hematopoietic stem cells, 761
Ion channels, stretch-activated, 239–240
Irgacure2959 (Ciba), 997
Iris pigment epithelium (IPE), 1148–1149
Irradiation, 75
ISC. *See* Intestinal stem cell
Ischemia, 1334–1335
Ischemia/reperfusion injury model, 829, 837, 1124–1125
Ischemic stroke, 1028
Islet graft, intracapsular environment and longevity of encapsulated, 672–673
Islet transplantation programs, 665–666
Isolation, definition of, 1564
Isoproterenol, 1454
ITOP. *See* Integrated tissue-organ printer
IUGT. *See* In utero gene therapy
IUTx. *See* In utero transplantation
IVB. *See* In vivo bioreactor
IVD. *See* Intervertebral disk (IVD)
Ixmyelocel-T, 777–778

J

Japan Pharmaceuticals and Device Agency, 1568
Jervell and Lange-Nielsen deafness syndrome, 1099
Jetting-based bioprinting, 1392–1394, 1403, 1404*t*
advantages, 1394
drawbacks of, 1394
Joint degeneration, articular cartilage injury, 968
Joint injuries, articular surface damage, 967
Joint pain, 967
Jordy, 1166–1167
Junctional epidermolysis bullosa (JEB), 1302

K

Kanamycin, 1094–1095
Karyotypic analysis, 1476
Keloids, 1318–1319
cellular and extracellular matrix characteristics of, 1319*t*
Keratan sulfate, 1138
Keratin, 321
Keratins 3, 1117–1119
Keratin 12, 1117–1119
Keratin15 (KRT15), 1293–1295, 1298
Keratin filaments, 1332
Keratinocyte differentiation-associated protein (Kdap) secretion, 1462
Keratinocytes, 99–101, 1214, 1289, 1291, 1294, 1299, 1301–1302, 1311–1312, 1332–1334, 1338–1341, 1483, 1485, 1545
Keratocan, 1138
Keratocytes, 1138–1139
Keratoepithelioplasty, 1118
Keratopathy, 1140
Kidney-on-a-chip, 832
Kidney tissue–derived primary or stem/progenitor cells, 826
primary renal cells, 826–828
stem/progenitor cells derived from kidneys
renal stem cells in Bowman's capsule, 828
renal stem cells in papilla, 828
renal stem cells in tubules, 828–829
Kidney, tissue engineering of, 825–826, 837–838
cell-based, 826–835
cell sources, 826–830, 834*t*
tissue-engineered cellular three-dimensional renal constructs, 830–835
cell-free
in situ kidney regeneration, 835, 836*t*
stromal cell–derived factor-1, 837
Kidney tubuloid, 815
Killer cell-immunoglobulin like receptors (KIRs), 773–774
Kinesins, 69
Klinefelter syndrome, 872
Knockout-DMEM (KO-DMEM), 425
Knockout Serum Replacement (KSR), 425
Kostmann syndrome, 778
Krt5-positive epithelium, 1261
Krt15 promoter, 1294
Kruppel-like factor 2 *(KLF2)*, 446
KSR. *See* Knockout Serum Replacement
Kupffer cells, 728–729, 1431–1432, 1540

L

Label-retaining cells (LRCs), 712, 828, 1289–1290, 1292
Lactate, 1462
Lactate dehydrogenase A *(Ldha)*, 1293
Lactide/glycolide grafts, 620
LacZ, 1294
LAMB3, 1302
Lamina propria, 845, 871
Laminar flow, 192
Laminin, 72–73, 97, 125, 226, 1031, 1311–1312, 1358
Laminin 332, 1312
Laminin-511, 100–101, 1139
Laminin-521, 1139
Laminin-containing gels, 1316
Laminin E8 matrix, 1138
Laminin protein, 1051, 1408–1409
Langerhans cells (LCs), 776–777, 1118, 1289, 1300, 1485
Large B-cell lymphoma, 770–771
Large cartilaginous airways, 1256
Large sacral pressure ulcer, 1343*f*
Large-scale cell culture, 1362
Large-scale food production, 1355
Large-scale production, 1370, 1372
Large-scale tissue culture, 1492
Laryngotracheal agenesis, 462–463
Laser-assisted bioprinting (LAB), 299–301, 305, 925*t*, 1394–1395, 1408–1409
Laser-based stereolithography, 1395
Laser-Doppler techniques, 1494
Laser etching, 1056
Laser-induced forward transfer (LIFT) cell printing technique, 997, 1395, 1398–1399, 1408
Laser-patterning, 1446
Laser photocoagulation, 1155
Laser skin, 1321
Latanoprost, 1153
Lateral plate mesoderm (LPM), 806–807

Later-stage development, regulatory process, market, 1559–1561
  current good manufacturing practice (cGMP), 1559
  pharmacology and toxicology, 1560
  Phase 3 clinical development, 1560–1561
  potency assay, 1560
  product readiness for Phase 3, 1559–1560
Layer-by-layer (LbL) assembly, of nanofilms, 281–282
L-dihydroxyphenylalanine (L-DOPA) therapy, 1025–1027
Least burdensome principles, 1563
Leber congenital amaurosis (LCA), 1173
Lecticans, 127–128
Left maxillary alveolar cleft, 1205*f*
Left-ventricular assist devices (LVADs), 1532
Leghemoglobin, 1361
LEH, 791
Lentivirus, 501–502, 538–539, 954, 1012–1013
  vectors, 501–502, 505, 507, 528
Lentivirus-based Survivin overexpression, 954
Lentivirus-transduced autologous hematopoietic stem cells, 502
Leptin receptor, 1011
Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5), 710–712, 1257, 1295, 1541–1542
Leucine-rich repeat-containing G-protein-coupled receptor 6 (Lgr6), 1257, 1295, 1297
Leucine-rich repeats and immunoglobulin-like domain protein 1 (Lrig1), 1295
Leucine zippers, 4
Leukemia, 769
Leukemia inhibitory factor (LIF), 468–469
Leveraging techniques, 742
Leydig cells, 871, 873–874, 874*f*
LFP. *See* Local field potentials (LFP)
Lichen planopilaris, 1299–1300
LIF. *See* Leukemia inhibitory factor
Life cycle analysis (LCA), 1364
Ligand receptor-based Turing model, 230–231
Ligand-receptor signaling, 83–85
  growth factor-b pathway, 83–84
  hedgehog pathway, 85
  Notch pathway, 85
  by receptor tyrosine kinase ligands, 84–85
  Wnt pathway, 84
Ligands, cell receptor, 523
Light-assisted bioprinting, 1421
  digital light processing (DLP)–based bioprinting, 1421
  two-photon polymerization (TPP)–based bioprinting, 1421
Limbal allograft transplantation, 1119
Limbal epithelial stem cell (LESC), 1115–1119, 1136–1137
Limbal stem cell deficiency (LSCD), 1117–1118, 1137
Limbal stem cell (LSC) niche, 1116–1117
  noncorneal sources as alternative to, 1119
  in ocular surface disease, 1118–1119
  positive and negative markers of, 1117*t*
  regulation of, 1116–1117
  and transient amplifying cells, 1116–1117
Lin28b, 812–813
Lineage-specific differentiation programs, 765
Lineage-tracing, 806–807, 917–918, 1260–1261
LinkN, 953
Lipid-based delivery systems, 536–537
Lipid-laden fat cells (adipocytes), 560
Lipid NPs (LNPs), 531
Lipid peroxidation, 1049–1050
Lipid soluble molecules, 73
Lipofectamine transfection, 955
Lipoprotein lipase deficiency (LPLD), 526–527
Liposomes, 530*f*, 536–537
Liquid-liquid flow, 192
Liver
  architecture and function, 723
  and cardiac organoids, 1452*f*
  development, 723–724
  lobules, 723
  physiological microfluidic models of, 742
  specification, 723–724
  transplantation, 726
Liver cancer disease, organoids as model to, 730
Liver constructs, three-dimensional, 741–742
Liver culture, two-dimensional, 741
Liver disease, cell-based therapies for, 738*f*
Liver disease burden, 737–738
Liver failure, 738, 769
Liver fibrogenesis, 1431–1432
Liver-on-a-chip, 1447
Liver organoids, 728, 1540
  novel scaffolds for, 729–730
Liver parenchyma, 740
Liver progenitor cells (LPCs), 724, 726–727, 730–731
  hepatocytes to, 730–731
  in organ regeneration, 724–726
Liver regeneration, 724–725
  hepatocytes role in, 725
  liver stem cells in, 725–726
Liver stem cells, in liver regeneration, 725–726
Liver therapies, 738–740
  biopharmaceuticals, 738
  clinical trials, 740
  extracorporeal liver support devices, 738
Liver tissue models, 1430–1432, 1431*f*
Liver transplantation, orthotopic, 723
Living bioreactor, 626
L-methionine, 1095–1096
lncRNA-Bmncr, 889
Load bearing bone defects, 927–929
Lobules, 557–558
Local field potentials (LFP), 1037–1041
Long bone segmental defects, 1010
Longevity, business issues, 1573–1574
Long telomeres, 848
Long-term hematopoietic stem cells (LT-HSCs), 757, 760
Long-term immunosuppression therapy, 863
Long terminal repeats (LTR), 500–501
Loop of Henle, 815, 826, 832–833
Low-energy shock wave (LESW) therapy, 876–877
Lower lip, massive squamous cell carcinoma of, 1206*f*
Lower urinary tract reconstruction, 845
Low-frequency *Cre*-inducible genetic model, 1291
Low-frequency electromagnetic fields (LF-EMF), 186
Low-risk devices, 1564
Low-viscosity materials, 1418–1420
Low vision enhancement system, 1166–1167
LPCs. *See* Liver progenitor cells
LRCs. *See* Label-retaining cells
LRME-independent uptake, 525
L-type channel blockers, 1097
Lumbricus terrestris erythrocruorin (LtEc), 792–793
Lumican-null mouse model, 1119
Lung
  branching morphogenesis in, 218–220, 220*f*
  neuronal innervation in, 219
Lung bud organoids, 1541
Lung epithelial progenitor/stem cells, 1260–1262
Lung microvasculature in lung repair, 1262
Lung on chip, 1265–1266, 1448
Lung organoids, 1541, 1543
Lung parenchyma, 1257, 1261, 1263, 1265
Lung regeneration
  induced pluripotent stem cells
    pulmonary disease, treatment of, 1263–1264, 1264*f*
    to pulmonary epithelial cell lineages, 1264–1265
  lung morphogenesis, 1254–1263, 1255*f*, 1256*f*
    diverse epithelial and mesenchymal cell types, 1256–1257
    embryonic development of alveolar capillaries, 1258–1259, 1259*f*
    endothelial progenitor cells in lung repair, 1262–1263
    lung epithelial progenitor/stem cells, 1260–1262
    lung microvasculature in lung repair, 1262
    pulmonary vasculature, structure and function of, 1257–1258, 1258*f*, 1260*f*
    signaling and transcriptional pathways, lung formation, 1256
  pulmonary cell-replacement strategies for, 1263–1265
Lung tissue engineering, 1273
  biofabrication approaches, 1274–1276
  bioreactor technologies, 1280–1281, 1281*f*
  decellularized scaffolds, 1274–1276
  design criteria for, 1273–1274, 1274*f*
  pulmonary endothelial engineering, 1277–1280
  endothelial seeding into lung scaffolds, 1278–1279

induced pluripotent stem cell derived endothelial cells, 1278
mesenchymal support of pulmonary microvasculature, 1280
organomimetic endothelial culture, 1279–1280
pulmonary endothelial cells, 1278
pulmonary epithelial engineering, 1276–1277
distal airway engineering, 1276–1277
mesenchymal support of pulmonary epithelium, 1277
proximal airway engineering, 1276
Lung tissues, bioengineering of, 1265–1266
conducting airways, tissue engineering for, 1266
extracellular matrix in lung structure and repair, 1265–1266
mesenchymal stromal cell products, 1265
mesenchymal stromal cells, 1265
pulmonary macrophage transplantation, interstitial lung disease, 1266
Lung tumor models, 1449
Lymphocytes, 557–558, 770
NK cells, 773–775
NKT cells, 775–776
T cells, 770–773
Lymphodepletion, 771–772
Lymphoid-lineage-derived DCs, 776–777
Lysyl oxidase (LOX) family enzymes, 122

M

M2 antiinflammatory macrophages, 777–778
Machine learning, 1040
of cellular dynamics, 47
in tissue engineering, 45–47
MACI, 1503–1504
MACIT. *See* Membrane-associated collagens with interrupted triple helices
Macrobioreactors, 179–191
Macrocapsules, 668
Macroengineering, 1055, 1055*f*, 1056*f*, 1057*f*
Macromolecules, conjugation of, 531
Macrophages, 364–365, 412, 620, 777–778, 890, 919–921, 1233, 1300, 1310, 1312–1313
activated, 404
intermediate, 1310
M1/classical, 1310
M2/nonclassical, 1310
monocyte-derived, 777–778
Macropinocytosis (MPC), 524
Macroporous hydrogels, 287
Macroporous pHEMA scaffolds, 1078–1079
Macroscopic heart valve, 635
Macular translocation surgery in AMD, 1170
Madin–Darby Canine Kidney (MDCK) cells, 82
Magnesium supplementation, 1096–1097
Magnetic field guided delivery, 1139–1140
Magnetic immunoselection, 479–480
Magnetic nanoparticles (MNPs), 18–19, 25–26
Magnetic resonance elastography (MRE), 191
Magnetic resonance imaging (MRI)
bone and cartilage reconstruction, 981, 981*f*
magnetic labeling, 761
Magnetic resonance spectroscopy (MRS), 191
Magnetoencephalography (MEG), 1037–1038
Maillard reaction, 1362–1363
Major histocompatibility complex (MHC) class I molecules, 776–777, 876–877, 889–890
Major histocompatibility complex (MHC) class II molecules, 776–777, 876–877
Malassez, 1225
Male reproductive organs
ejaculatory system, 874–875
engineering vas deferens, 874
spinal ejaculation generator, 875
penis, 875–877
penile reconstruction, 875–876
penile transplantation, 876
stem cell therapy for erectile dysfunction, 876–877
testes, 871–874
androgen-replacement therapy, 873–874
spermatogonial stem cell technology, 871–873
Mammalian cells, 75
Mammalian lung, cleft formation in, 219*f*
Mammalian stretch activated ion channels, 239–240
Mammary gland, development, 567*t*
Mandibular defects, 1207*f*, 1215–1216
anterior lateral thigh fasciocutaneous and fibula osteocutaneous vascularized free flaps, 1207*f*
avascular bone graft, placement of, 1206*f*
lower mandible, resection, 1207*f*
massive squamous cell carcinoma of the lower lip, 1206*f*
nonvascularized grafts, 1206–1207
vascularized grafts, 1205–1206
Mandibular distraction osteogenesis (MDO), 1008, 1009*f*
Mandibulectomy, 1511
Manufacturer's working cell bank (MWCB), 1488–1490, 1492
MAPK, 244–245, 809–810
Marfan syndrome, 126, 637
Markers for corneal cell types, 1137*t*
Marrow-derived stem cells (MSCs), 260–261
calcium sensing receptor (CaSR) in, 263–264
Marrow-stimulation techniques, 1500
Masquelet technique, 1514
Massive mandibular defect, image-guided tissue-engineered reconstruction of, 1516*f*
Massive squamous cell carcinoma of lower lip, 1206*f*
Masson trichrome, 1312*f*
Mass transport, 167, 181–184, 407–408
Mastectomy, 558–559
Master cell banks (MCBs), 1488, 1555
Material–cell interactions, 351
Material-mediated gene delivery, 538
Mathematical models, 37
to clinical reality, 47–48
Matricellular protein, 103, 205
interactions and activities, 206*t*
multifunctional, 126–127
Matricryptins, 102
Matrigel, 831*t*, 832, 1263, 1358, 1403–1406, 1423*t*, 1448–1449, 1524, 1540–1541
-coated culture, 1122–1123
ovarian cancer cells on, 1401–1403
Matrisome, 1265–1266
Matrix and cell injuries, 970–971
Matrix-assisted autologous chondrocyte implantation (MACI), 892–893
Matrix-based regenerative therapies, 1233
Matrix bound thrombospondin, 1316
Matrix carboxyglutamic acid protein (MGP), 44–45
Matrix-induced autologous chondrocyte implantation, 979
Matrix metalloproteinase (MMP), 4, 18–19, 81, 301, 353, 892, 948, 1214, 1319, 1334, 1427, 1524
MMP-1, 1311–1312, 1314, 1317
MMP-3, 948–952
MMP-7, 101
MMP-8 (collagenase-2), 1314
MMP-13, 1314, 1317, 1463
MMP-14, 1262
Matrix mineralization, 888
Matrix molecules, 103–104
degradation of, 112
Matrix-P, 1527
Matrix-P Plus, 1527
Matrix proteins, 102
Matrix receptors, 107
Mature collagen molecules, 121
Mature-fabricated models, 1425
Maxillary alveolar clefts, 1205*f*, 1208
Maxillectomy, 1511
Maxillofacial trauma, 1203
M1/classical macrophages, 1310
Mayer–Rokitansky–Küster–Hauser syndrome (MRKHS), 863
M210-B4 stroma, 774–775
M cells, 709–710
MCY-M11, 777–778
Meat processing technology, 1381
Meat production, 1355–1356
Meat, tissue engineering of, 1357*f*. *See also* Food tissue engineering
consumer acceptance, 1364–1365
enhanced meat, 1363
scale, 1361–1362
Mechanical forces, in biological systems, 237–238, 238*f*
Mechanical heart valves, 638–639
Mechanical loading of articular surfaces, 967
Mechanical prosthetic valves, 638–639
Mechanical transduction complex (p130), 1008
Mechanical valves, 1526
Mechanobiology, 266–267
bone-implant design, 248–250
defined, 237

Mechanobiology (*Continued*)
in tissue engineering, 248–252
Mechanoelectrical transduction channels, 1094
Mechanosensing, cellular, 238–243
Mechanotransduction, 246*f*, 1318, 1380
in biological phenomena, 245–248
cellular effects of, 243–245
of larger tissues, 238–239
multitude of, 245
Medical device
Breakthrough Devices Program, 1563
definition of, 1563
least burdensome principles, 1563
regenerative medicine advanced therapy, evaluation of devices used with, 1564
risk-based approach, 1563
Medical implants, 317–319, 333–334
biodegradable biomaterials in, 319*t*
Medical procedure code, 1581
Medical product testing, cell-based products, 1568
Medicare, 1574, 1581–1582
Medulla, 683
Medullary development and expansion, 696
Medullary thymic epithelial cells (mTECs), 683, 687–688, 694–696
MEF. *See* Mouse embryonic fibroblast
MEF2 family, 148
Megakaryocytes, 769–770, 769*f*
Meiotic cycles, 74
Melanin, 1332
Melanocytes, 1332, 1486–1487
Melanoma, 770
Melanoma epitope MART-1-specific CTLs, 773
Melanopsin, 1174–1175
Melatonin, 836*t*
Memantine, 1098
Membrane-associated collagens with interrupted triple helices (MACIT), 123
Membrane-bound beta glycan, 103
Membrane-bound IL-21, 774
Membrane-bound Kit ligand (mKITL), 683
Membrane-guided bone regeneration therapy of osseous defects, 1209
Membrane-type matrix metalloproteinase-2 (MT2-MMP), 229
Menaflex, 900–901
Meniere's disease, 1103
Meningomyelocele (MMC), 461
Meniscus, 900–901
Menopause, 558
Mercury-based amalgams, 1187
MERTK, 1147–1148
mESC. *See* Murine embryonic stem cells
Mesenchymal cells, 79, 81, 93–94, 217, 1191, 1273–1274
vs. epithelial cells, 80*f*
Mesenchymal-epithelial transition (MET), 79, 82–83, 446
Mesenchymal precursor cells (POMPs), 1140
Mesenchymal progenitor cells, 1011
Mesenchymal stem cells (MSCs), 7, 111, 138, 164, 179, 407, 539*f*, 643–644, 825, 883, 889, 917–919, 926, 948, 972, 980, 994, 999, 1011, 1124, 1138–1139, 1194, 1211–1212, 1359–1361, 1403–1406, 1429–1430, 1459, 1472
adult and fetal stem cells, 829
biology, 883
bone tissue engineering, 897–898
cardiac resident, 584–585
cartilage tissue engineering, 891–897
cells for, 892–897
general properties of, 892
for cell therapy in eye, 1124
gene therapy in, 901
heterogeneity, 889
host immunobiology, effect on, 889–891
identification, 883–884
isolation and in vitro culture, 886–887
in musculoskeletal tissue engineering, 891
osteochondral tissue engineering, 898–899
plasticity of, 888–889
safety of, 891
secretome of, 995–996
self-renewal and proliferation capacity, 887–888, 887*f*
skeletal tissues with, 899–901
meniscus, 900–901
tendon/ligament repair, 899–900
skeletogenic differentiation of, 888
sources of, 994–995
surface markers, 884–885
tissue sources of, 885–886, 885*t*
Mesenchymal stromal cells (MSCs), 345–346, 429, 767, 1265
as hemophilia A therapeutics, 383–385
preclinical success with, 384–385
products, 1265
Mesenchymal support/pulmonary epithelium, 1277
Mesenchymal support/pulmonary microvasculature, 1280
Mesenchymal-to-epithelial transition (MET), 810–811
Mesenchyme, 76, 230, 1189
Mesoangioblast, 150–151
Mesoderm, 806
Mesonephros, 807
Messenger RNAs (mRNAs), 148–149
MET. *See* Mesenchymal-epithelial transition
Metabotropic glutamate receptor (mGluR6), 1176
Metacarpophalangeal joint arthroplasties, 973
Metalloproteinases (MMPs), 636–637
Metal oxides, 18
Metanephric mesenchyme, 808
Metanephric nephrogenesis, 810*f*
Metanephros, 805–806
Metastasis, 248
metHb, 787–788, 791–792
Methotrexate, 1431–1432
3-methyladenine (3-MA), 1095
Methylation, DNA, 68
Methylcellulose, 729–730
Methylprednisolone, 1073–1076
MGP. *See* Matrix carboxyglutamic acid protein
Mi2beta, 767–768
Micelles, 534
Micro- and nanospheres, 1073
Microarray, bioreactors, 478*f*
Micro-biofabrication, 1445–1446
Microbioreactors, 179, 191–197
design principles, 191–194
flow rheology, 191–193
Microbubble-enhanced ultrasound gene therapy, 954–955
Microcapsules, 669
Microcolumns of autologous skin for autologous grafting, 1322
Microcontact printing, 1446
Microelectrode arrays, 1037–1038
Micro-electromechanical system analog model, 1102
Microelectronic technologies, 1443
Microencapsulation, 669
Microengineered mesothelioma tumor organoids, 1543–1544
Microengineering, 1056, 1057*f*, 1446–1447
Microenvironmental signals, 557–558
Microextrusion deposition, 925*t*
Micro-extrusion printing techniques, 982–983
Microfabrication approaches, 476, 742–743, 983*f*, 1443–1444, 1447–1448
Microfibrils, 126
Microfilament-engineered cerebral organoids, 1425–1426
Microfilaments, 69–70, 70*f*
Microfluidic-based systems, 17–18
Microfluidic chambers, 1265–1266
Microfluidic design, 1541–1542
Microfluidic device, 196, 760, 1403
Microfluidics, hydrogel fabrication with, 194
Microfluidic technologies, 477–478, 898, 1077–1078, 1443–1444, 1447–1448, 1450
Microfracture technique, 973, 985
Microgravity, effects of, on bone, 190
Microgravity bioreactor, 189–190
Microhemorrhages, 1310
Micromixers, passive, 192
Micron-sized particles, 522
Microparticles, 896–897
Micropatterned cocultures (MPCCs), 741
Micropatterning, 1446
Microperforations, basement membrane, 228–230
Micro-piezoelectric technology, 1102
Micropumps, 192
Microreactors
components and integration into, 194–195
design principles for, 191*f*
perfusion-based, 194
types, 194
MicroRNAs, 135, 437, 890, 955, 1012–1013, 1256
in branching morphogenesis, 225–226
regulators of differentiation, 148–149
Microscale 3D weaving technique, 162
Microscale bioreactors, 1448–1449
Microscale fluidic systems, 1443–1444

Microscale technologies, 476
Microspheres, 407–408
  polymer, 286
Microsurgical techniques, 1209–1210
Microtissues, 1239–1241
Microtubules, 69, 70*f*, 239
Microvalves, 192
Microvascular angiogenesis, 1275–1276
Microvascular endothelial cells, 1277–1279
Microvesicles derived from stem cells, 929
Mild NPDR, 1155
Milk production by tissue-engineered, 1363–1364
Millipore Sigma, 1470
Mimic neo-organogenesis ex vivo, 1372
Mineral-based scaffolds, 1015
Miniaturization technologies, 191–192
Minimally invasive arthroscopic surgery, 1501
Minimally invasive surgery, 1209
Minocycline, 1049, 1073–1076
miR-140-5p, 955
miR-141, 955
miR-188, 889
Mitochondrial calcium uniporter (MCU), 1094
Mitochondrial pyruvate carrier *(Mpc1)*, 1293
Mitogenic activities, 1315–1316
Mitotic activity of lung parenchyma, 1257
Mitral valve prolapse, 637
MMC. *See* Meningomyelocele; Myelomeningocele
MMLV. *See* Moloney murine leukemia retrovirus
M2/nonclassical macrophages, 1310
MNPs. *See* Magnetic nanoparticles
Moderate nonproliferative DR, 1155
Modified chitosans, 940–942
Modified hemoglobin (Hb) species, 785
Moist wound healing in chronic wounds, 1334
Molecular farming, 1470
Molecular signals, 145
Molecular transport, 183
Moloney murine leukemia retrovirus (MMLV), 500
Monocyte-derived dendritic cells, 776–777
Monocyte-derived dendritic cells-based vaccines, 776–777
Monocyte-derived macrophages, 777–778
Monocytes, 620, 770, 890, 919, 1310
Monogenetic congenital diseases, 1493
Monolayer tissue growth, in vitro, 42
Monomers, polymers derived from, 534–536
Monomethoxy poly(ethylene) glycol, 505
Morphogen, gradient effects, 134–135
Morphogenesis, 37, 41, 44–45, 93, 809, 1322–1323
Morphogens, 134–135
  as bioactive signaling molecules, 134–135
  as signaling cues in tissue engineering, 140
Motion program generation, 1391–1392
Motogenic activities, 1315–1316
Mouse embryonic fibroblast (MEF), 468–469
  spontaneous differentiation of, 469
Mouse embryonic fibroblasts, 425
Mouse embryonic stem cells (mESCs), 445, 467
MP4OX, 790–791
MPC. *See* Macropinocytosis
MPC1cE, 891
MRE. *See* Magnetic resonance elastography
MRFs. *See* Myogenic regulatory factors
MRKHS, 867
MRS. *See* Magnetic resonance spectroscopy
MRSA-infected tibial nonunion, 1515*f*
Msh homeobox 1 (Msx), 1298
MT2-MMP. *See* Membrane-type matrix metalloproteinase-2
Mucocutaneous junctions, composite grafts for, 1214–1215
Mucoperiosteal flap, 1231
Müller glia, 1146, 1154
Müllerian duct malformations, 863
Müller stem cells, 1121
Multicellular cerebral cortex model, 1541*f*
Multicellular tissues, 79
Multidomain peptides (MDPs), hydrogels, 4
Multidrug-resistant strains of pathogenic bacteria, 1369–1370
Multifunctional matricellular proteins, 126–127
Multilineage differentiating stress enduring (MUSE) cells, 888–889
Multilineage differentiation, manipulation of, 760
Multiorganoid devices, 1544, 1544*f*
Multiple body systems, in tissues, 137*f*
Multiple myeloma, 1342, 1343*f*
Multiple sclerosis, 1124
Multiple transgenes, 164–165
Multipolymeric nanoparticulate approach, 535
Multipotency, hair follicle stem cells, 1294
Multipotent adipose-derived stem cells (MADS), 1360
Multipotent progenitor cells, 714–715, 917–918
Murine amphotropic envelope, hybrid proteins of, 505
Murine chondrocytic ATDC5 cells, 43*f*
Murine embryonic stem cells (mESC), 468–469
Murine experiments, with in utero transplantation, 372
Murine HF stem cells, markers of, 1293*t*
Murine progenitors, markers of, 1293*t*
Muscle cell differentiation, 150*f*
Muscle cells, safe differentiation media, 1377–1378
Muscle-derived mesenchymal stem cells, 508–509
Muscle-derived stem cells (MDSCs), 1011
Muscle fibers, 146–147, 149–150, 152, 1378
Muscle myocytes, 560
Muscle regeneration, 147, 149–151
Muscle repairing, 150–151
Muscle-specific transcription factors, 147
Muscle–tendon unit (MTU) construct, 1407–1409
Musculoskeletal tissue engineering, mesenchymal stem cells (MSCs), 891
Musculoskeletal tissues, 157
Mutagenesis
  genomic integration associated insertional, 385–386
  insertional, 495, 502
Mutual cross-repression, 38–40
Mutual learning, 1040
Myelin debris, 1051
Myelin disorders, 1031
Myeloablative chemotherapy, 779
Myeloblasts, 1448–1449
Myelodysplastic syndrome, 757–758
Myeloid angiogenic cells (MACs), 1125
Myeloid-derived EPC-like cells, 1263
Myeloid-lineage leukocytes, 777–778
Myeloid lineage progenitors, 765
Myelomeningocele (MMC), 369
Myf-5, 147, 149, 151
Myoblast culture, 1377
Myoblasts, 147, 149, 1372–1375, 1378, 1380
Myocardial infarction (MI), 1016, 1428–1430, 1527
Myocardial signaling, 580*f*
Myocardial tissue, 1356–1357
Myocardium, 157
  electromechanical effects on engineered, 171
Myocyte enhancer factor-2 (MEF2) proteins, 148
Myocytes, muscle, 560
MyoD, 147–148
  expression of, 147
Myofibroblast, 104, 110, 207, 585*f*, 643, 1138, 1194, 1317, 1333
  apoptosis, 104
  arterial, 643
  differentiation, 103
Myogenesis, 148, 150
Myogenic regulatory factors (MRFs), 147–148
Myogenin, 147–148
Myoglobin, 1359
Myointimal hyperplasia, 618–619, 623
Myometrium cells, 864
Myosatellite cells, 1376–1378
Myosin inhibitor (blebbistatin), 1121
Myotubes, 1359
Mysenchymal stem-cell (MSC), 1221
  from gingival tissues, 1222–1223

N

N-acetyl cysteine (NAC), 738, 1095–1096
N-acetyl-*m*-aminophenol, 1449
NAFLD. *See* Nonalcoholic fatty liver disease
Nail, 1296–1297
Nail stem cell (NSC) niche, 1296–1297
Naive human embryonic stem cells, 424
Nanocarriers, 522
  PEGylated, 522–523
Nanocomposite polymers, 18, 926
Nanofiber, 759–760, 997–998
Nanofibrillated cellulose, 984–985
Nanofibrous scaffolds, 352, 895, 899

Nanofilms, layer-by-layer (LbL) assembly of, 281–282
Nano-fluid chambers, 565
Nanoparticles, 18, 519, 1077*f*, 1103, 1395–1396, 1447–1448
  biodistribution of, 522
  chitosan, 535–536
  efficiency of, 524*t*
  inorganic, 537
  neutrally charged, 522
  physicochemical properties of, 525
  polymer, 284, 286
  scaffold of, 1585
  skin-penetrating, 537
  titanium oxide, 18
Nanoparticulate shape variation, 526*f*
Nanosilver–PLGA composite BMP-2 scaffolds, 927–929
Nanotechnology, 13, 757
  cultured meat technology, 1375
  integration of, 18–19, 25–26
Nanotopography, 929–930
Nanowoven fibers, 1015–1016
NASH. *See* Nonalcoholic steatohepatitis
Nasoseptal chondrocytes, 1500–1501
National Institute for Health and Care Excellence (NICE), 1499
National Institutes of Health (NIH), 1337
National Institutes of Standards and Technology (NIST), 1568
Native bone, 249
Native cartilage, 159
Native extracellular matrix, 1056–1058, 1065
Native tendon scaffolds, 998–999
Native tissues, 1418
Natural and synthetic polymer scaffolds, 1047
Natural biomaterials, 640–641
Natural bone grafts, 897
Natural collagen matrix, 851–854
  acellular tissue matrices, 852
  collagen, 852
  matrix binding with growth factors, 852–854
  silk fibroin (SF), 852
Natural extracellular matrix, 835
Natural killer (NK) cells, 374–375, 773–775, 889–890
Natural killer-92 (NK-92), 774–775
Naturally derived biomaterials, 864, 1421, 1423*t*
Naturally derived materials, 562–563
Naturally derived polymers, 562, 1058
Natural materials, 459–460
Natural polymers, 186, 297–298, 298*t*, 535–536, 670, 831*t*, 832
Natural polymers, biomaterial, 1058–1072
  extracellular matrix polymers, 1058–1065
  polymers derived from the blood, 1071–1072
  polymers from marine/insect life, 1065–1070, 1068*f*, 1069*f*, 1070*f*
Natural scaffolds, 1015
Natural silk fibroin, 1069–1070
Natural tissue regeneration, 1191–1192, 1192*f*
Natural tissue regeneration, 1191
N-cadherin, 80, 1117–1118, 1140, 1446
NCCs. *See* Neural crest cells
NCLX (sodium calcium exchanger), 1094
Necrosis, 16
  coagulative, 593–594
Negative regulators of development, 148–149
Nemo Binding Domain peptide, 953
Neobone formation, 263–264, 927
Neochondrogenesis, 1212–1213
Neodermis, 1335
Neofolliculogenesis, 1298
Neogenic HFs, 1298
Neomycin, 1094–1095
Neonatal cardiac repair, 582–583
Neonatal heart, cardiac regeneration in, 584*f*
Neonatal immortalized keratinocytes (NIKS), 1322
Neonatal stem cells, 457
Neonatal tissue engineering, scaffolding specifics in, 459–460
Neotissue development, in tissue engineered heart valves, 645–646
Neovagina reconstruction, 866–867
Neovascularization, 17–18, 25
Nephrogenesis, 812–814
Nephron equivalents, 817
Nephron progenitor cells (NPCs), 809–816
Nephrostomy tube, 847–848
Nerve autografts, 1052
Nerve growth factor (NGF), 1028, 1051
Nerve injuries, 1426–1427
Nervous system, 137–138
Nestin expression, 1011
Netarsudil, 1153
Neural crest cells (NCCs), 97, 581, 685–686, 690, 1138, 1140
Neural-like tissues, 883
Neural progenitor cells, 472
Neural regeneration, in animal models, 480
Neural relays, 1426–1427
Neural stem cells (NSCs), 1059, 1076, 1424–1425
Neural tissue, 1409
Neurite outgrowth inhibitor A (NOGO-A), 1051
Neuroblastoma, 775–776
NeuroD, 147–148
Neuroendocrine cells, 1256
Neuroepithelial bodies (NEBs), 1256
Neuroepithelial cells, 480
Neurogenic bladder dysfunction model, 847–848, 854
Neurogenins, 147
Neurological Disorders and Stroke (NINDS), 1169–1170
Neurologic toxicity, 770–771
Neuronal activity, 1380
Neuronal lipoid fuscinosis, 7
Neuronal precursor cell (NPC) type, 428–429
Neuron depolarization, 1175
Neuron hyperpolarization, 1175
Neuropathic diabetic foot ulcers (DFUs), 1340–1341
Neuropilin 1 (NRP-1), 1258, 1278
Neuroprostheses, 1037, 1039–1041, 1041*f*
Neurotrophic factor neurturin (NRTN), 221
Neurotrophic factors, 1052, 1093, 1098–1099
Neurotrophin 3 (NT-3), 1051, 1069*f*, 1098, 1103
Neurotrophin-4/5 (NT-4/5), 1172
Neurotrophin-501, 1154
Neurotrophin-503 implant, 1172
Neurotrophin therapy, 1100
Neutralizing Abs (NAbs), 379
Neutropenia, 778
Neutrophil collagenase (MMP-8), 1317
Neutrophil extracellular traps, 1191–1192
Neutrophils, 364, 620, 778–779, 919–921, 1310
Neutrophils bind several matrix molecules, 100–101
New stroma, 1312–1313
Next generation approaches, 1275–1276
Next-generation biomanufacturing platform systems, 1472–1473
NHP. *See* Nonhuman primate
Nicardipine, 1097
Nicotinamide, 774
Nicotinamide-expanded CB stem cells (NiCord), 778
Nimodipine, 1097
Nitric oxide, 1096
NKT cells, 775–776
NKX2-1, 1254–1255, 1256*f*
Nocturnal bruxism, 1202–1203
Nodal signaling, 1121–1123
Noggin, 1296
Noise-induced apoptosis shifts, 1097
Noise-induced hearing loss (NIHL), 1096–1098
Nonalcoholic fatty liver disease (NAFLD), 737–738
Nonalcoholic steatohepatitis (NASH), 737–738
Nonallergenicity of biomaterial, 1358–1359
Nonbiodegradable hydrogels, 1074*t*, 1077–1080
Noncoding RNAs, 83, 1256
Noncollagenous domains, 122–123
Noncontractile cardiac patches, 1527–1528
Nondegradable polymers, 561–562
Nondestructive technologies, 1459
Nonhematopoietic cells, 883
Non-Hodgkin lymphoma, 770–771
Nonhuman collagen, immunogenicity of, 459–460
Nonhuman primate (NHP), 370, 385
Noninferiority studies, 1506–1507
Nonintegrating minicircle plasmids, 1012–1013
Noninvasive electroencephalographic (EEG), 1037–1038
Nonischemic murine neurodegenerative diseases, 1125
Non-label-retaining cell, 1290–1291
Nonmesodermal MSC-derived cells, 883
Nonobstructive azoospermia, etiology of, 871–872

Nonocular stem cells
bone marrow stem cells, 1124–1126
endothelial colony-forming cells, 1125–1126
hematopoietic stem cells/CD34^{+} cells, 1124, 1125*f*
mesenchymal stem cells, 1124
potential for stem cells in ocular repair and tissue engineering, 1126
retinal disease, 1124–1125, 1125*f*
embryonic stem cells in retinal regeneration
photoreceptors from, 1122–1123
retinal ganglion cells from, 1123
retinal pigment epithelial from, 1121–1122
induced pluripotent stem cells (iPSCs) in retinal regeneration, 1121, 1122*f*
hematopoietic/vascular progenitors (CD34/endothelial colony-forming cells), 1123–1124
photoreceptors from, 1122–1123
retinal ganglion cells from, 1123
retinal pigment epithelial from, 1121–1122
Nonparenchymal cells (NPCs), 723
Nonproliferative DR (NPDR), 1155
Nonscarring alopecias, 1299
Nonsyndromic hereditary hearing loss, 1093
Nontoxicity of biomaterial, 1358
Nonvascularized grafts, soft and hard tissue defects, 1205–1207
Nonviral gene delivery, 954–955
of nucleic acids, 540
formulations, 530*f*
from scaffolds, 540
vectors, 1210–1211
vehicles, 531
Nonviral nucleic acid delivery, 522, 525–526, 530–531
Nonviral transfection, 901
Nonviral vectors, 497, 522
endocytosis of, 523
Normal tension glaucoma (NTG), 1153
Normann microelectrode, 1174
Northern white rhinoceros (NWR), 452
Notch2 receptor, 811
Notch activity, 810–811
Notch intracellular domain (NICD), 713
Notch ligand Jag1, 1300
Notch pathway, 85, 713
Notch proteins, 713
Notch signaling, 776, 1261
Notochordal cells, 956
Novocart, 947, 1504
Novocart Basic, 1503
Novocart Inject, 1504
NovoGen Bioprinter platform, 1431–1432
NpHR, 1175, 1177
NPs. *See* Nanoparticles
NR4A1 (fibrosis inhibitor), 954–955
NRTN. *See* Neurotrophic factor neurturin
NSAIDs, 1155, 1454
NT-3 delivery, 1103
N-terminal of native bFGF (NAT), 864
Nuclear chromosomes, 65–66
Nuclear DNA, 66
Nuclear export sequence (NES), 83
Nuclear factor of activated T cells c1 (NFATc1), 1293
Nucleic acid-based drugs, 519
Nucleic acid delivery, 519
nonviral, 530–531
for tissue engineering, 541
viral, 526–530
Nucleic acids, nonviral delivery of, 540
Nucleotide bromodeoxyuridine (BrdU) labeling technique, 828
Nucleotide-free recombinant protein, 1012–1013
Nucleus pulposus (NP), 938, 938*f*, 940–948, 945*f*, 952, 955
Nurr transcription factor, overexpression of, 471–472
Nutrition, cellular, 404–405
Nutrition-related diseases, 1369–1370
NXAUTO, 1469

O

Ocular morphology in healthy and diseased conditions, 1165*f*
Ocular tissues, 1163
ODNs. *See* Oligodeoxynucleotides
Odontoblasts, 1187–1190, 1192–1194, 1196
Office of Communication, Outreach and Development (OCOD), 1557
Office of Compliance and Biologics Quality (OCBQ), 1559
Office of Tissues and Advanced Therapies (OTAT), 1553, 1557, 1568
Off-label use, 1016, 1341–1344, 1514–1516
Off-the-shelf product, 1477
Ohm's law model, 567–568
OI. *See* Osteogenesis imperfecta
Oligodendrocyte precursor cell (OPC) delivery, 1059–1065
Oligodendrocytes, 472–473, 1171
Oligodendroglial cells, 480
Oligodeoxynucleotides (ODNs), 519
Oligonucleotide modifications, 531
Oligo(poly(ethylene glycol)-*co*-fumarate), 1208–1209
Oligo(poly(ethylene glycol) fumarate) (OPF), 1212
Oncomodulin, 1172
OP1, 1016
OP-1, 894
OP9, 772
OP9 coculture method, 777–779
Open flap debridement (OFD), 1229–1230
Optical and electronic image enhancement techniques, 1166–1167
Optical coherence tomography (OCT), 1463
Optical matter, 1375
Optical systems, 1167
Optic nerve protection and regeneration, 1171–1172
Optimal cell manufacturing process, 189
Optimal transport, 446
Optogenetic approaches, 1177
Optogenetics, 1174–1177
Oral and maxillofacial region, definition of, 1201, 1202*f*
Oral and maxillofacial surgery
animal models, 1215–1216
avascular bone graft, placement of, 1206*f*
bone applications, 1208–1212
BMP-2, 1209
direct technique, 1211
gene transfection, 1211
growth factors, 1209–1210
hemi-mandible, regeneration of, 1210*f*
indirect delivery methods, 1211
mesenchymal stem cells, 1211–1212
minimally invasive surgery, 1209
muscle-flap prefabrication technique, 1209–1210
nonviral gene delivery systems, 1211
oligo(poly(ethylene glycol) fumarate), 1212
recombinant protein-based strategies, 1210–1211
rhBMP-2, 1209–1210
scaffolds, 1208–1209
secondary-staged tissue-engineered graft, 1210*f*
surface chemistry, 1212
cartilage applications, 1212–1214
challenges in, 1201–1204
composite tissue applications, 1215
facial symmetry, 1204
fractured atrophic mandible, 1204*f*
future of, 1216
left maxillary alveolar cleft, 1205*f*
mandibular defects, 1205–1207
anterior lateral thigh fasciocutaneous and fibula osteocutaneous vascularized free flaps, 1207*f*
avascular bone graft, placement of, 1206*f*
lower mandible, resection, 1207*f*
massive squamous cell carcinoma of the lower lip, 1206*f*
nonvascularized grafts, 1206–1207
vascularized grafts, 1205–1206
maxillary defects, 1207–1208, 1207*f*
oral and maxillofacial region, definition of, 1201, 1202*f*
oral mucosa applications, 1214–1215
osteosarcoma of maxilla, 1202*f*
self-inflicted gunshot wound
with avulsive injury, 1203*f*
maxillofacial skeletal structures, disruption and loss of, 1204*f*
squamous cell carcinoma, floor of mouth and tongue, 1203*f*
Oral cancer, 1511
Oral mucosa applications, oral and maxillofacial surgery, 1214–1215
Orcell, 1493
Organ donors, 1
Organ engineering, 8–9, 24, 460
Organize cells, molecules that, 79–82
Organ of Corti, 1100

Organogenesis, 259, 1337
historical context, 259–260
Organoids, 2, 13–19, 436, 873
bile duct-derived, 728
hepatocyte-derived, 728–729
intestinal, 717
as model to liver cancer disease, 730
Organomimetic endothelial culture, 1279–1280
Organ-on-a-chip (OOC) technologies, 2, 17–18, 196, 250–252, 817, 832, 1445–1449
cancer-on-a-chip, 1448–1449
cell manipulation in, 251*t*
heart-on-a-chip, 1448
liver-on-a-chip, 1447
lung-on-a-chip, 1448
microengineering and biofabrication, 1446–1447
personalized precision medicine, 1454–1455, 1455*f*
strengths and limitations of, 195*t*
tissue organoid models and applications, 1544, 1544*f*
vessel-on-a-chip, 1447–1448
Organotypic culture, 926, 1339
Organotypic vasculature engineering, 265–267
Organ printing, cultured meat technology, 1375
Organ regeneration
hepatocytes in, 724–726
liver progenitors in, 724–726
Organs, 77–78
decellularized, 24
Organ-specific stem cell progenitors, *vs.* pluripotent stem cells, 714–715
Organ transplant preservation, 794–795
Oriented and lamellar electrospun polycaprolactone (PCL) fibers, 943
Orthopedic reconstruction, articular cartilage products, tissue engineering of, 1500
Orthotopic liver transplantation, 723
Osr1, 811–812
Osseointegration, of bone implants, 249
Ossification, 264–265
OssiMend, 1233–1234
Osteoarthritis (OA), 887*f*, 1499
Osteoarthrosis, gene therapy for, 541
Osteoblast cells, 301
Osteoblastic VICs, 636
Osteoblast-like cells, 480–481
Osteoblasts, 919
Osteochondral autografts and allografts, 1500
Osteochondral autograft transplantation surgery, 985
Osteochondral constructs, 1215
Osteochondral defects, 930–931, 972–973, 1499–1500
Osteochondral injuries, 970–974, 970*t*, 985
Osteochondral repair, 164
Osteochondral tissue engineering, 898–899, 985
Osteochondral unit, 307*f*
Osteochondritis dissecans (OD), 1506
Osteochondrorecticular cells, 1011
Osteoclast-mediated remodeling, 919–921
Osteoclasts, 921–922
Osteoconductivity, 1014–1015
Osteocytes, 888–889, 925–926
Osteogenesis, 888, 897–899, 924*t*, 925–929, 1014, 1227
Osteogenesis imperfecta (OI), 463
Osteogenic cells (OCs), 474, 1007
Osteogenic cytokines, 1007
Osteomyelitis, 1514
Osteon, 1235
Osteonecrosis, 898
Osteo-odonto-keratoprosthesis, 1167
Osteopontin, 209–210
function of, 210
phenotype of, 210
proinflammatory and profibrotic properties of, 210
Osteopontin-derived peptide, 1212
Osteoporosis, 898
Osteoporotic fractures, 1007
Osteoporotic models, 929
Osteosarcoma, 1515–1516
of maxilla, 1202*f*
Osteosynthesis, in bone IVB, 263*f*
Oswestry Disability Index (ODI), 953
OTAT Learn, 1554
Ototoxic drugs, 1094
Ototoxicity, 1096–1097
prevention of, 1094–1096
Ototoxic medications, 1093
Otx2, 1151
Outer hair cells (OHCs), 1094, 1097
Outer limiting membrane (OLM), 1146, 1150–1151
Outer nuclear layer (ONL), 1123, 1146–1147
Outer plexiform layer (OPL), 1146
Outer retinal cell transplantation, 1177
Outer root sheath (ORS), 1294–1295
Outflow tract (OFT), 579
Ovarian cancer cells, on Matrigel matrix, 1401–1403
Ovarian cell encapsulation techniques, 866
Ovarian follicles, tissue engineering, 866
Ovary, female reproductive organs, 865–866
Ovine critical-sized defect model, 929
Oxidative stress, 1094, 1096
inhibitors, 1155
OxiVita, 790
Oxycyte, 793–794
OxyFluor, 793–794
Oxygen, 1461, 1463–1464
delivery, 789
equilibrium curve, 789*f*
Oxygenation, 1444
of immunoprotected cells, 409–410
Oxygen bridge, 794
Oxygen-induced retinopathy model, 1156
Oxygent, 793–794, 794*f*
Oxygen tension, 895
Oxyglobin, 790
OXYVITA Inc, 790

P

p63 (Trp63), 1260–1261
Pain visual analog scale (VAS), 953
p-AMPKa, 1094
Pan American Health Network for Drug Regulatory Harmonization (PANDRH), 1568
Pan-caspase inhibitor ZVAD, 1097
Pancreas, transplantation of, 665
Pancreatic cancer organoids, 1542–1543
Pancreatic organoid cultures, 1542
Pancreatic progenitors (PPs), 657–659
Paneth cells, 711*f*, 712–713
Papain-dissociated photoreceptors, 1150–1151
Papilla, renal stem cells in, 828
Paracrine effects, 849–850, 1377–1378
Paracrine hypothesis, 585
Paracrine signaling, 991–992, 1259
Parallel Scientific Advice or Consultative Advice, 1568
Parasympathetic ganglion (PSG), 220–221
development in salivary gland, 222*f*
Parathyroid hormone (PTH), 263–264
Parathyroid organogenesis, 689–696
molecular control, 689–691
molecular regulation of, 689–696
specification, 692–695
Parenchymal cell migration, 1324
Parkinson's disease (PD), 423, 450, 452, 472, 480, 1025, 1030*f*, 1031, 1589
dopaminergic function, 1025
fetal transplantation therapy for, 1026–1027
stem cell replacement strategies, 1026–1027
Partial-chondral defects, 1499–1500
Partial pneumonectomy, 1262
Partial uterine repair
cell-seeded scaffolds for, 864–865
scaffold-free approaches for, 865
Particulated autologous/allogenic articular cartilage, 1503
Particulated Juvenile Allograft Cartilage (PJAC), 1503
Passive micromixers, 192
Patellofemoral joint, 969
Pathogen-associated molecular patterns (PAMPs), 670, 1310
in polymers, 670
Pathological scars, 1318–1319
Patient-specific cardiomyocytes generation, 609, 610*f*
Patient-specific cell therapy, 1424–1425
Patient-specific products, 1356
Pattern formation, 44–45, 45*f*
Pattern-recognition receptors (PRRs), 670
Pattern-recognition techniques, 1040
Pax, in development, 149
Pax3, 149
Pax7, 149–151
P-cadherins, 1312
PCL. *See* Polycaprolactone
Pedicled flap, 1207–1208
Pegaptanib (Macugen), 1147
Pegylated G-CSG (Pegfilgrastim), 778

PEGylated nanocarriers, 522–523
PEGylation, 522–523, 529–530
PEGylation of LtEc, 794
Pelizaeus-Merzbacher disease, 7, 1027
Penile cancer, 876
Penile vibratory stimulation (PVS), 875
Penis, 875–877
  penile reconstruction, 875–876
  penile transplantation, 876
  stem cell therapy for erectile dysfunction, 876–877
Pentoxifylline, 1096–1097
Peptide ligands, immobilizing, 350–351
Peptide nucleic acids (PNAs), 531
Peptides
  cell-penetrating, 535
  fusogenic, 535
  and proteins, 318–322
Perfluorocarbon (PFC), 793–795, 794*f*
Perftoran, 793–794
Perfused bioreactors, 159–160
Perfusion-based microreactors, 194
Perfusion bioreactors, 16–17
Perfusion decellularization, 1525
Perichondrial grafts, 973
Pericytes, 926, 1194
Periimplantitis, 1229–1230
Perinatal stem cells, 458–459
Periodontal ligament (PDL), 1221–1223
Periodontal ligament cell (PDLC), 1223, 1226–1227
Periodontal ligament stem cells (PDLSCs), 1223–1224, 1226–1227, 1227*t*
Periodontal tissue engineering and regeneration, 1221–1222
  biomaterials science, 1232–1236
    biomaterial-based immune modulation, 1233
    biomaterial redesign, 1235–1236
    ceramic-based materials, 1235
    classes of biomaterials, 1233
    naturally derived polymers, 1233–1234
    requirements of cell scaffolds, 1232–1233
    synthetic polymers, 1234–1235
  cell scaffolds, requirements of, 1232–1233
  challenges and future directions, 1242
  signaling molecules, 1227–1232
    bone morphogenetic proteins, 1228
    crucial delivery barriers to progress, 1230–1231, 1231*f*
    enamel matrix derivative, 1229–1230
    fibroblast growth factor-2, 1228–1229
    gene delivery, alternative to growth factor delivery, 1231–1232
    growth/differentiation factor-5, 1229
    platelet-derived growth factors, 1228
    platelet-rich plasma, 1229
    stem cell–derived exosomes, 1230
    types of signals, 1228–1230
  stem cells, 1222–1227
    adipose-derived stem cells, 1226
    bone marrow–derived mysenchymal stem cells, 1225–1226
    cell types, selection of, 1226–1227, 1227*t*
    from dental pulp, 1225
    exfoliated deciduous teeth, 1225
    extraoral mysenchymal stem cells, 1225
    Hertwig's epithelial root sheath (HERS), 1225
    intraoral mysenchymal stem cells, 1222–1223, 1223*f*
    periodontal tissue–derived stem cells, 1223–1224
  strategies
    cell-based approaches, 1237*f*, 1239–1242, 1240*t*
    cell-free approaches, 1237–1239, 1237*f*, 1238*f*, 1239*f*
Periodontitis, 1221–1222, 1239
Periosteal cells, 264
Periosteal flap, 892–893
Periosteal grafts, 973
Periosteum, 262–263, 926, 1224
Peripheral arterial disease (PAD), 620–621, 1526
  vascular pathology in, 621*f*
Peripheral blood (PB), 765
Peripheral blood mononuclear cells (PBMNCs), 849–850
Peripheral cochlear implant, 1102–1103
Peripheral nerve injuries, 1426–1427
Peripheral nerve regeneration, 334
Perlecan, 128
  expression of, 98–99
Peroxiredoxin 3 (Prx3), 1095
Personalized medicine, 2, 17, 21, 1543*f*
Personalized precision medicine, organ-on-a-chip, 1454–1455, 1455*f*
Peyer's patches, 709–710
Pfirrmann's classification, 947–948
PGA. *See* Polyglycolic acid
PGC. *See* Primordial germ cells
PGs. *See* Proteoglycans
Phage display technology, 523
Phagocytosis, 523–524, 778
Pharmaceutical agents, 160–161
Pharmaceutical Inspection Convention and Pharmaceutical Inspection Cooperation Scheme (PIC/S), 1568
Pharmacoeconomics
  definition of, 1580
  types of, 1580
Pharmacologic studies, tissue engineering as a platform for, 611–612
Pharmacology and toxicology, 1555–1556, 1558, 1560
Pharyngeal pouch outgrowth, transcription factors and regulation of, 691–692
PHAs. *See* Polyhydroxyalkanoates
pHEMA-*co*-methyl methacrylate (pHEMA-MMA), 1076–1077, 1079–1080
PHH. *See* Primary human hepatocytes
PHHs. *See* Primary human hepatocytes
Phosphorylated receptors, 73
Photoacoustic imaging (PAI), 191
Photochemical bonding, 159–160
Photo-crosslinkable bioinks, 300
Photo-crosslinkable polymeric resins, 1399
Photo-curing-based techniques, 1395–1396
Photodiode arrays, 1168
Photodynamic therapy (PDT), 1172
Photo-initiator, 1395, 1397
Photolithography techniques, 1056–1058
Photo-patterning, 1446–1447
Photopolymer, 1395
Photopolymerizable biomaterials, 1421
Photopolymerization
  for printing, 1421
  property of PEG hydrogels, 742–743
Photoreceptors, 1146, 1150–1151, 1163–1164, 1166, 1170–1173, 1177
  from pluripotent stem cells, retinal degeneration, 1151–1153
  in retinal regeneration, 1122–1123
Photosensitive structures, 1174, 1175*f*
Photo-stereolithography, 897
Photosystem I (PSI), 1174
pH, tissue engineering systems, 1461
Physical forces, 161
Physical signals, 477–478
Physiological microfluidic models, of liver, 742
Piezoelectric inkjet bioprinters, 1392–1394, 1418–1419
Piezoelectric polymer films, 284
Pilocarpine, 1153
Piribedil, 1098
Piston-based dispensing system, 1394
Pixium, 1168
Placenta, 458
Placenta-derived stem cells, 848
Plasma membrane, 71
Plasmid, 497, 852–854, 954–955
  DNA, 497
  gene delivery, 525–526
Plasminogen activating system, 1317
Plasminogen activator, 1316
Plasmonic gold nanorods, 308
Plasticity, definition of, 888–889
Platelet-derived growth factor (PDGF), 134, 208–209, 477, 540, 836*t*, 888, 948, 949*t*, 971, 1011, 1014, 1209, 1228, 1280, 1333, 1485
Platelet-derived growth factor (PDGF)-BB, 1309, 1312–1313
Platelet factor-4 (PF-4), 1315
Platelet lysate, 887
Platelet-rich plasma (PRP), 953, 995, 999, 1210–1212, 1221, 1225–1226, 1229
Platelet-rich plasma-impregnated gelation hydrogel microspheres (PRP-GHMs), 952–953
Platelets, 769–770, 769*f*
PLET1, 687
Plethora of synthetic bone graft materials, 930
Pluripotency, 37
Pluripotent cells, metabolism of, 424
Pluripotent stem cells (PSCs), 421, 458, 586–588, 714–715, 717, 723, 726–728, 727*f*, 767, 772, 777–779, 826, 1027–1028, 1115, 1120, 1137,

1149–1150, 1171, 1538, 1540, 1578–1579
advantage of using, 581
cardiac development with, 581–582
derivation of cardiomyocytes from human, 599–601
kidney, tissue engineering of
embryonic stem cells, 829–830
induced pluripotent stem cells, 830
megakaryocytes/platelets, 769–770, 769*f*
nigral dopaminergic differentiation protocols of, 1028
vs. organ-specific stem cell progenitors, 714–715
red blood cells, 765–768
beta-thalassemia, 766–767
fetal hemoglobin, 766–767
induced PSCs (iPSCs), 767
Mi2beta, 767–768
sickle cell disease, 766–767
reprogramming of somatic cells into induced, 445–446
retinal pigment epithelium, 1149–1150
screens, induced, 438*t*
white blood cells, 770–779
granulocytes, neutrophils, 778–779
lymphocytes, NK cells, 773–775
lymphocytes, NKT cells, 775–776
lymphocytes, T cells, 770–773
monocyte-derived dendritic cells, 776–777
monocyte-derived macrophages, 777–778
Pluripotent stem cells-derived kidney organoids, 816*f*
Pluronic F-127, 1394, 1397–1398, 1422, 1432–1435
PMCs. *See* Primary mesenchyme cells
PNAs. *See* Peptide nucleic acids
Pneumatic-based dispensing system, 1394
Pneumonectomy, 1260
Podocytes, 816–817, 828
Podoplanin (Pdpn), 1258
Point-of-care method, 1512–1513, 1515–1517
Policymakers, 1590
Polyacrylamide (PAM), 997–998
Poly α-hydroxy acid polymers, 1073–1077
Poly(α-hydroxyesters), 897–898
Poly(alkylacrylic acids), 525
Poly(amino acids), 332–333, 562
Polyanhydrides, 562
Polycaprolactone (PCL), 459, 562, 832, 850, 993, 1208–1209, 1234
Polycarbonates, aliphatic, 330, 330*f*
Polyclonal VDJ recombination, 772
Polycomb-group (PcG) proteins, 757
Poly(dimethylsiloxane) device, 760
Polydimethylsiloxane microfluidic chips, 1434–1435
Poly(ε-caprolactone) (PCL), 162, 297, 329, 329*f*, 1076, 1394, 1422–1424
Poly-ε-caprolactone fumarate (PCLF), 1076
Poly-ε-caprolactone (PCL) polyethylene oxide, 895
Polyesters, 297–299
aliphatic, 561
Polyesters of glycolic acid (PGA), 1072–1073
Polyesters of lactic acid (PLA), 898–899, 1072–1073
Polyetherurethane/poly-L-lactide (PU/PLLA), 864
Poly(ethylene glycol) (PEG), 522, 561–562, 758–759, 895, 940–942, 1072–1073, 1076, 1078*f*, 1234, 1404*t*, 1406–1407, 1422, 1524
Poly(ethylene glycol) (PEG)-based bioinks, 1422
Poly(ethylene glycol) (PEG)-based hydrogels, 163–164, 1398
Poly(ethylene glycol) diacrylate (PEGDA), 993, 1395–1397, 1404*t*, 1408, 1422
Poly(ethylene glycol) dimethacrylate hydrogel, 1406
Poly(ethylene glycol) hydrogels, 1446
Poly(ethylene glycol) methacrylate (PEGMA), 1422
Poly(ethylene glycol) (PEG) surface conjugated Hb (PEG–Hb), 789–791
Polyethylene terephthalate (PET), 622
Polyethylene tubes, 874
Polyethylenimene (PEI), 531
Poly(glycoamidoamine)s (PGAAs), 536
Poly(glycolic acid) (PGA), 318, 327–329, 327*f*, 459, 561, 831, 850, 857, 864, 895, 940–942, 980, 993, 1208–1209, 1212–1214, 1234
Polyglycolic acid (PGA)-based scaffolds, 856
Polyglycolic acid–collagen cell-seeded scaffolds, 857–858
Polyglycolic acid (PGA) grafts, 625
Polyglycolic acid/poly-L-lactic acid (PGA/PLLA), 716
PolyHeme, 790
Poly(histidine) (poly(His)), 535
Polyhydroxyalkanoates (PHAs), 318, 325–326
Poly(3-hydroxybutyrate) (PHB), 325–326
Poly(2-hydroxyethyl methacrylate) (pHEMA), 981–982, 1077–1078, 1079*f*
Polyhydroxyoctanoate (PHO), 642
Poly(lactic acid) (PLA), 297, 831, 930, 993, 1208–1209, 1212–1213, 1399
Poly(lactic-*co*-glycolic acid) (PLGA), 162–163, 791, 794, 831, 850, 864, 866–867, 898, 980, 997, 1010–1011, 1013–1015, 1055, 1073–1076, 1077*f*, 1211, 1229, 1399, 1425–1426, 1484, 1502, 1524
Polylactide (PL), 327–329, 327*f*, 561, 1234–1235
Poly (D,L-lactic acid), 1234–1235
Poly(L-lactic acid) (PLLA), 345, 459, 475, 895, 1234–1235
Poly-L-lysine (PLL) coating, 412
Poly(Lys), 535
Polymeric controlled-release devices, 1030
Pegaptanib (Macugen), 1147
Polymerase, RNA, 67
Polymer-based delivery systems, 525–526
Polymer brushes formation, 671–672
Polymer chemistry, on cell behavior, 280–284
Polymer-derived products, 282
Polymer fibers, 1058
Polymeric hemoglobin, cross-linked and, 790
Polymeric hydrogels, for three-dimensional bioprinting, 13
Polymeric scaffolds, 1052
Polymerization, 8–9
of styrene, 284
Polymer microspheres, 286
Polymer nanoparticles, 284, 286
Polymer polycaprolactone (PCL), 1069–1070
Polymers, 530*f*, 921*t*
of aryloxyphosphazenes, 332
biodegradable, 282, 317–318, 325
biologically derived, 318–326, 333
cationic, 533–534, 536
cell interactions with, 275–285
to create tissue engineered products, 333–334
degradable, 282
derived from blood, 1071–1072
derived from natural sources/monomers, 534–536
electrically charged, 284
electrically conducting, 284
electrically conductive, 348
implantation of, 287–288
marine/insect life, 1065–1070, 1068*f*, 1069*f*, 1070*f*
nanocomposite, 18
natural. *See* Natural polymers
naturally derived, 562
nondegradable, 561–562
pathogen-associated molecular patterns in, 670
protein adsorption to, 280
surface chemistry of, 281
synthetic. *See* Synthetic polymers
synthetic derived, 333
Polymer scaffold fabrication, 295–297
cell types and biological interactions, 300–301
cell viability and activity assessment, 301–302
design inputs, 297–302
extrusion printing, 304–305
inkjet printing, 303–304
laser-assisted bioprinting, 305
materials and inks, 297–299
methods of, 296*t*
open source and commercial 3D printing systems, 306–307
porous architecture, 309
printing applications, 310
printing/patterning of multiple inks, 308
print outputs, 307–309
print resolution, 308–309
processing and cell viability, 299–300
scaffold fidelity assessment, 309
stereolithography, 305–306
3D printing systems and printer types, 302–307, 303*f*, 304*t*
Polymersomes, 530*f*

Poly(methyl methacrylate) (PMMA) cement spacers, 930, 1514
Poly[*N*-(2hydroxypropyl)methacrylamide] (pHPMA), 1080
Poly(*N*-isopropylacrylamide) (pNIPAM), 333
Polynitroxylated α−α cross-linked Hb (VitalHeme), 790−791
Polynucleotides, 326
Poly(ortho esters), 329−330
Poly(*p*-dioxanone) (PDS), 329
Polyphosphazenes, 331−332
  biodegradable, 332
Polyplexes, 532−533
Poly(propylene fumarate) (PPF), 1208−1209, 1399, 1403−1406
Polypropylene mesh, 564
Polysaccharides, 322−325, 323*t*, 670
Polytetrafluoroethane (PTFE), 459
Polytetrafluoroethylene (PTFE), 622−623, 864
Poly(trimethylene carbonate) (PTMC), 317
Polyurethane, 758−759
Polyurethane bioink, 1425
Polyurethanes, 1208−1209
  biodegradable, 330−331
Population doublings (PDs), 846
Porcine muscle progenitor cells, 1376
Porcine TMJ disk cells, 1213
Porous calcium phosphate cement, 926
Porous hydroxyapatite (HA) granules, 1224
Porous PEG-based polymers, 1076−1077
Porous poly(methyl methacrylate) (PMMA), 1212
Positive feedback-based molecular switches, 38−40
Positively charged OPF + , 1078*f*
Postmitotic photoreceptor precursors, 1177
Postnatal cells, 458
Post-pneumonectomy response, 1260
Posttraumatic osteoarthritis (OA), 967−968, 972
  risk of, 968
Posttraumatic synovitis, 974
Potency assay, 1560
Potent chemoattractants, 620
Powered pumps, 192
PPQ. *See* Process performance qualification
PPROM. *See* Preterm prelabor rupture of membranes
PPs. *See* Pancreatic progenitors
Preadipocytes, 560
Precision medicine (PM), 196−197
Pre-descemet's membrane (DM), 1135−1136
Premarket applications (PMAs), 1561−1562
Premature rupture of membranes (PROM), 369−370
Prenatal therapy, 460
  immunology, 460
  physiology, 460
Presbyacusis, 1093
Press-coating process, 898−899
Pressure ulcers, 1342
Preterm prelabor rupture of membranes (PPROM), 369−370
Pretubular aggregate (PTA), 810−811
Primary adult cells, 1537−1538
Primary cells, 405−406
Primary dentinogenesis, 1195
Primary fissures penetrating calcified cartilage, 969
Primary human hepatocytes (PHHs), 726, 741
Primary inflammatory disorders, 1048
Primary intestinal-derived organoid units, 716−717
Primary mesenchyme cells (PMCs), 79
Primary mode of action (PMOA), 1561
Primary open-angle glaucoma (POAG), 1153
Primary renal cells, 826−828
Primary tissue brain implants, 1025−1027
Primitive stem cells, 848
Primordial germ cells (PGC), 386
Printing cartridges, 1392
Print resolution, 308−309
Priority Review, 1564−1565, 1564*t*, 1565*t*
Private payers, reimbursement, 1580
Process performance qualification (PPQ), 56−57
Product development (PD), 53−56, 1577−1580, 1577*f*
  direct reprogramming of differentiated cells, 1580
  embryonic stem cells, 1578−1579
  induced pluripotent stem cells, 1579−1580
  small molecule-induced differentiation, 1580
Proepicardial cells, 580
Progenitor cells, 710−712
  endothelial, 473
  intestine with, 714−717
  neural, 472
  origin of, 579−581
  vascular, 473
Progenitors
  pancreatic, 658−659
  ventricular cardiomyocyte, 581
Progenitor VICs, 636
Programmed cell death (apoptosis), 100, 1049−1050, 1315
Proinflammatory cytokines, 898, 919, 953
Projection stereolithography, 897, 1275−1276
Project management (PM), 58
Proline hydroxylation, 121
PROM. *See* Premature rupture of membranes
Proneural genes, 147
Proof-of-concept (POC), 866−867, 1025, 1554−1555, 1578
Prophylactic therapy, 1095−1096
Propidium iodide, 301−302
Propranolol, 1454
Proprioception, 238
Prostacyclin, 1277−1278
Prostaglandin analogs, 1153
Prostaglandin D_2 (PGD_2), 1299−1300
Prostaglandin D_2 synthase (PTDGS), 1138
Prostaglandin E_2 (PGE_2), 890, 1213
Prosthetic appliances, 1208
  alloplastic reconstructions with, 1205
Prosthetic heart valves, 638
Prosthetic valves, mechanical, 638−639
Protein, stability, 83
Protein adsorption, to polymers, 280
Proteinases, 1313*f*
Protein coding genes, 66
Protein expression, 40
Protein kinase A (PKA), 73−74
Protein-protein interactions, 4
Proteins
  Adsorbed, synthetic polymers with, 282−283
  extracellular matrix (ECM), 4, 24, 119
  intracellular, 69
  matricellular, 103, 205
  matrix, 102
  myocyte enhancer factor-2 (MEF2), 148
  peptides and, 318−322
  synthetic, 284
  therapeutic, 405
  therapy, 629
  transmembrane, 80
  uptake and secretion of, 1462
Proteins-based smart biomaterials, 21−22
Proteoglycan-rich microdomains, 942*f*
Proteoglycans (PGs), 127−128, 322, 892−894, 938, 944, 948, 968−969, 971−972, 990
  degradation, 94
  heparan sulfate (HS), 227−228
  hyaluronan-binding, 127−128
  leucine-rich repeat, 128
  TGFb-binding, 99
Proximal airway engineering, 1276
Proximal interphalangeal joint arthroplasties, 973
Proximal tubular epithelial cells, 828
PRRs. *See* Pattern-recognition receptors
Pseudophakic bullous keratopathy, 1139
Pseudo-poly(amino acids), 332−333
Pseudotyping, 529
PSG. *See* Parasympathetic ganglion
Psoriasis, 1301
Psoriatic arthritis, 1202−1203
PTFE. *See* Polytetrafluoroethylene
PTH. *See* Parathyroid hormone
Public Health Service (PHS) Act, 1473, 1553
Pulmonary alveolar proteinosis (PAP), 777−778, 1266
Pulmonary artery (PA), 1278−1279, 1279*f*
Pulmonary edema, 1277−1278
Pulmonary embolism, 1434−1435
Pulmonary endothelial cells, 1278
Pulmonary endothelial engineering, 1277−1280
  endothelial seeding into lung scaffolds, 1278−1279
  induced pluripotent stem cell derived endothelial cells, 1278
  mesenchymal support of pulmonary microvasculature, 1280
  organomimetic endothelial culture, 1279−1280
  pulmonary endothelial cells, 1278
Pulmonary epithelial cell lineages, induced pluripotent stem cells, 1264−1265
Pulmonary epithelial engineering, 1276−1277
  distal airway engineering, 1276−1277

Pulmonary epithelial engineering (*Continued*)
mesenchymal support of pulmonary epithelium, 1277
proximal airway engineering, 1276
Pulmonary epithelium, mesenchymal support of, 1277
Pulmonary hypertension, 209–210
Pulmonary macrophage transplantation, interstitial lung disease, 1266
Pulmonary microvasculature, mesenchymal support of, 1280
Pulmonary surfactant, 1274
Pulmonary transplantation, 777–778
Pulmonary vasculature, 1258
structure and function of, 1257–1258, 1258*f*, 1260*f*
Pulmonary vein (PV), 1278–1279, 1279*f*
Pulpal inflammation, 1191–1192
PVDF-based intracochlear microphone prototype, 1101–1102
Pyoderma gangrenosum, 1342
Pyrogenic contaminants, 670

Q

Quality-adjusted life years (QALYs), 1356
Quality assurance (QA), 53, 55, 58
Quality control (QC), 53, 1472–1473, 1488–1489
Quantum cell expansion system, 1476–1477
Quantum-on-demand bioprinting, 569–570
Quiescence, hair follicle stem cells, 1292–1293

R

Radial forearm osteocutaneous vascularized flap for intraoral soft tissue coverage, 1211*f*
Radiation therapy, 1205–1207
Radical mastectomy, 558
Rainbow reporter system, 1291
Random forests (RFs), 46
Ranibizumab, 1147
Rapamycin, 836*t*, 1049, 1095
Rapid cycling progenitor pool, 1290–1291
Reactive nitrogen species (RNS), 1095–1096
Reactive oxygen species (ROS), 787–788, 1094–1097
Readiness potential, 1039
Reaggregation FTOC (RFTOC), 684
Rebound hyperfusion, 1096
Recalcitrant ulcers, 1309
RECELL, 1321
Recellularization techniques, 825, 834*t*, 865, 1529
Receptor for hyaluronan mediated motility (RHAMM), 107, 127–128
Receptor-interacting protein (RIP) 3 siRNA modulate noise-induced necrosis, 1097
Receptor-mediated endocytosis, 523
Receptor tyrosine kinase (RTK), 96, 135, 808–809
Recombinant hemoglobin, 792
Recombinant human BMP-2 (rhBMP-2), 927, 952, 997, 1015–1016, 1209–1212, 1228, 1514
Recombinant human BMP-7 (rhBMP-7), 927, 929, 1516–1517, 1516*f*
Recombinant human BMP 12 (rhBMP-12), 999
Recombinant human FGF-2 (rhFGF-2), 1009, 1016
Recombinant human GDF-5 (rhGDF-5), 952
Recombinant human GDF-6 (rhGDF-6), 952
Recombinant human OP-1 (rhOP-1), 952
Recombinant NT-3 injection, 1073
Recombinant proteins, 1209–1211, 1377–1378
Recoverin, 1151
Recovery, definition of, 1564
Red blood cells (RBCs), 765–768
beta-thalassemia, 766–767
fetal hemoglobin, 766–767
induced PSCs (iPSCs), 767
Mi2beta, 767–768
sickle cell disease, 766–767
substitutes
complications of, 785
cross-linked and polymeric hemoglobin, 790
encapsulated hemoglobin, 791
erythrocruorins, 792–793
Hb-based O_2 carriers (HBOCs), 785–787, 787*f*
Hb extravasation, 788*f*
hemoglobin, sources of, 791–792
hemoglobin toxicity, 787–788
organ transplant preservation, 794–795
oxygen delivery, 789
oxygen equilibrium curve, 789*f*
oxygen offloading plot for Hb, 789*f*
perfluorocarbons, 793–794
recombinant hemoglobin, 792
replicating red blood cell functions, 785, 786*f*
surface conjugated hemoglobin, 790–791
viscosity and colloid osmotic pressure, 789–790
Red blood cell substitutes
complications of, 785
cross-linked and polymeric hemoglobin, 790
encapsulated hemoglobin, 791
erythrocruorins, 792–793
Hb-based O_2 carriers (HBOCs), 785–787, 787*f*
Hb extravasation, 788*f*
hemoglobin, sources of, 791–792
hemoglobin toxicity, 787–788
organ transplant preservation, 794–795
oxygen delivery, 789
oxygen equilibrium curve, 789*f*
oxygen offloading plot for Hb, 789*f*
perfluorocarbons, 793–794
recombinant hemoglobin, 792
replicating red blood cell functions, 785, 786*f*
surface conjugated hemoglobin, 790–791
viscosity and colloid osmotic pressure, 789–790
Redox imbalance, 1095–1096
Reepithelialization, 102–103, 1310–1312, 1321
Reepithelializing porcine wound, histology of, 1312*f*
Regenerative medicine (RM), 1, 40–41, 46–48, 53–54, 805, 825, 845–846, 871, 883, 889
Alliance for Regenerative Medicine, 54
biomanufacturing for
academic clinical center, advancing at, 1478*t*
approved products, 1474*t*
cell banking advance, 1477
challenges in, 1470–1472, 1471*t*
closed-modular biomanufacturing system, 1476–1477
current landscape of, 1469–1470
current workflows for, 1470, 1471*t*
medical applications, 1477–1478
off-the-shelf product, 1477
preservation advances, 1477
regulatory challenges for, 1473–1476
space exploration, 1478
synthetic biology advance, 1477
industry sectors, 1577*f*
risk of, 1575–1577
and tissue-engineered products, 1562–1563, 1562*f*
safety and effectiveness, for cell-scaffold, 1562*f*
3D bioprinting, 1563
in vitro studies to tissue engineering and, 159–160
Regenerative medicine advanced therapy (RMAT), 1322, 1473, 1522*f*, 1530–1532, 1553, 1564
evaluation of devices used with, 1564
Regenerative medicine therapies (RMTs), 1154, 1511, 1553
Regulatory process, market
cell-based products in medical product testing, 1568
clinical investigator responsibilities, 1567
clinical research conducted outside of United States, 1568
clinical research involving children, 1566
combination products, 1561–1562
development and approval process, 1554
early-stage development, 1554–1557
chemistry, manufacturing and controls, 1555
clinical, 1556–1557
pharmacology and toxicology, 1555–1556
expanded access to investigational drugs for treatment use, 1566
expedited review programs, 1564–1565, 1564*t*, 1565*t*
human cells, tissues, and cellular and tissue-based products (HCT/Ps), 1565–1566
homologous use, 1566

minimal manipulation, 1565–1566
investigational new drug (IND) application, 1557–1559
chemistry, manufacturing, and controls information, 1558
clinical protocol, 1559
cross-reference authorization, 1559
original investigational new drug application submission, 1559
pharmacology and toxicology data, 1558
previous human experience, 1558
required US FDA forms, 1557–1558
later-stage development, 1559–1561
current good manufacturing practice (cGMP), 1559
pharmacology and toxicology, 1560
Phase 3 clinical development, 1560–1561
potency assay, 1560
product readiness for Phase 3, 1559–1560
laws, 1553–1554
medical device
Breakthrough Devices Program, 1563
definition of, 1563
least burdensome principles, 1563
regenerative medicine advanced therapy, evaluation of devices used with, 1564
risk-based approach, 1563
OTAT Learn, 1554
sponsor–investigator responsibilities, 1567
sponsor responsibilities, 1567
standards, use of, 1568
tissue-engineered and regenerative medicine products, 1562–1563, 1562*f*
safety and effectiveness, for cell-scaffold, 1562*f*
3D bioprinting, 1563
US Food and Drug Administration international regulatory activities, 1568
US Food and Drug Administration/sponsor meetings, 1557
Regulatory T cells (Tregs), 770–772, 849–850, 889, 1124, 1300
Reimbursement, business issues, 1580–1582
Rejection pathways, 404
Relaxed (R) quaternary state, 785–787, 787*f*
Remineralization processes, 1191–1192
Remodeled stem cells, 1301
Remodeling, epigenetic, 446
Remyelination of axons, 1058
Renal failure, 805
Renal stem cells
in Bowman's capsule, 828
in papilla, 828
in tubules, 828–829
Renal transplantation, 805
ReNeuron, 1028, 1152–1153
Reparative dentinogenesis, 1192, 1192*f*
Replace, Reduce, and Refine (3Rs) principle, 190–191
Replication-deficient retroviral vector, 1289–1290
Replicative senescence, 887–888
Repopulating ischemic/diabetic retina, 1178
Reporter-based gene expression imaging, 1463, 1463*f*
RepRap system, 306
Reproductive medicine, 863
Reprogrammed hepatocytes, 744
Reprogramming techniques, 446–447
Residual biomaterial, 1358
Respiratory distress syndrome (RDS), 1256
Ret expression, 809
Reticulocytes, 767
Reticuloendothelial system, 521–522
Retinal cell transplantation, 1170–1171, 1174
Retinal degeneration, 1156–1157
age-related macular degeneration, 1147, 1148*t*, 1149*f*
prevalence, 1147
severity, 1147
wet/exudative form, 1147
diabetic retinopathy, 1155
cell-based therapies, 1148*t*
stem cell–based therapies, 1155–1156
eye and cellular layers of retina, 1146*f*
glaucoma, 1148*t*, 1153–1154
cell-based therapies, 1148*t*
stem cell–based therapies to, 1154–1155
photoreceptors from pluripotent stem cells, 1151–1153
retinal degenerative diseases, cell types affected in, 1145–1147
retinal pigment epithelium
as cellular therapy for age-related macular degeneration, 1147–1149
from pluripotent stem cells, 1149–1150
retina, structure/function of, 1145–1147
retinitis pigmentosa, 1148*t*, 1150–1151
visual impairment and blindness, epidemiology of, 1145
Retinal degenerative diseases, cell types affected in, 1145–1147
Retinal diseases, 1124, 1125*f*, 1170–1171
Retinal ganglion cells (RGCs), 1123, 1146–1147, 1154–1155, 1164–1166, 1171–1172, 1177–1178
in retinal regeneration, 1123
Retinal implants, 1178
stimulation, cortical response of, 1179*f*
Retinal ischemia-reperfusion injury, 1124
Retinal neurons, 1164
Retinal pigmented epithelium (RPE) cells, 423–424, 426–428, 438, 1027, 1120–1122, 1145–1150, 1163–1164, 1166, 1170–1171, 1173, 1177, 1578
as cellular therapy for age-related macular degeneration, 1147–1149
isolation of, 427–428
from pluripotent stem cells, 1149–1150
in retinal regeneration, 1121–1122
Retinal progenitor cells (RPCs), 1120–1121, 1152–1153
Retinal remodeling, alterations during, 1165*f*
Retinitis pigmentosa (RP), 1147, 1148*t*, 1150–1151, 1163–1164
Retinoblastoma (RB) tumor suppressor proteins, 692–693
Retinoic acid (RA), 807, 1028, 1151
signaling, 689, 1254–1255
Retroviral vectors, 500–501, 528
gene transfer strategies for, 501
targeting of, 505
Retrovirus, 499–500
Reverse transcription-polymerase chain reaction, 474
RGD-modified hyaluronan hydrogels, 1323
RHAMM. *See* Receptor for hyaluronan mediated motility
rHb0.1, 792
rHb1.1, 792
rHb3011, 794
rHBOC, 792
rHb (rHb3011) with glycine fused a subunits, 792
Rheometer, UV-compatible, 300
Rhesus (Rh) blood group system, 765
Rheumatoid arthritis (RA), 1202–1203, 1500
gene therapy for, 541
Rhodopsin, 1151
Rho GTPases
localization of, 243, 245*f*
promigratory regulation of, 107
Rho Kinase (ROCK) inhibitor, 1538
rhPDGF-BB, 952
Riabetic retinopathy (DR), 1145
Rib perichondrial arthroplasties, 973
Rifampin-bonded gelatin-sealed Dacron grafts, 628
Right maxillectomy
benign odontogenic neoplasm, removal of, 1206*f*
RISC. *See* RNA-induced silencing complexe
RM. *See* Regenerative medicine
RMAT designation program, 1564*t*, 1565, 1565*t*
RNA-induced silencing complex (RISC), 148–149, 519
RNA polymerase, 67
RNA sequencing (RNA-Seq), 1293
RNA viruses, 500
Robotic technology, 1472–1473
ROCK inhibitor, coinjection of cells with, 1139–1140
Rofecoxib (Vioxx), 1454
Root formation, 1190–1191
Rotary cell culture systems, 471
Rotating bioreactors, 167
Rotating wall perfused vessel (RWPV), 189–190
RPE. *See* Retinal pigment epithelium
R-phenyl isopropyl adenosine, 1096
RTKs. *See* Receptor tyrosine kinases
Runt-related transcription factor 2 (Runx2), 893–894
RWV bioreactors, 190

S

Saccharide-based materials, 534–535
Safe differentiation media, muscle cells, 1377–1378
Safranin-O staining, 1406, 1464

Salicylate, 1095–1096
Salivary gland
  branching morphogenesis in, 220–222, 221*f*
  parasympathetic ganglion (PSG) development in, 222*f*
Sall1, 807
Sandwich technique, 1506, 1508
Sarcomere, 145–146
Sarthran, 1096–1097
SATB2, 1013
Satellite cells, 149–150, 1357–1359, 1381
  in skeletal muscle differentiation and repair, 149–150
SBS. *See* Short bowel syndrome
Scaffold-based tissue-engineered vascular grafts, 1526
Scaffold-based tissue models, 1417–1418, 1420
Scaffold fabrication technique, 186–188, 832
Scaffold-free approaches, uterus, female reproductive organs, 865
Scaffold-free bioprinting method, 1400, 1432–1434
Scaffold-free cell printing, 1399–1400
Scaffold-free grafts, 1526
Scaffold-free three-dimensional systems, 1502
Scaffold-free tissue models, 1417–1418, 1420
Scaffold-mediated vector delivery systems, 164–165
Scaffolds, 275, 561–564, 850, 921*t*, 922–923, 991–992, 1208–1209, 1212, 1214, 1320, 1396, 1417–1418, 1484
  anisotropic, 9–10
  articular cartilage products, tissue engineering of, 1501–1502, 1501*t*
    acellular, scaffold-based products, 1503
    agarose and alginate, 1502
    autologous chondrocyte implantation, clinical generations of, 1503
    collagen scaffolds, 1501–1502
    hyaluronan, 1502
    particulated autologous/allogenic articular cartilage, 1503
    scaffold-free three-dimensional systems, 1502
    synthetic polymers, 1502
  biological tooth replacement and repair, 1189–1190
  biomimetic, 297
  bone tissue engineering, 1512
  cardiovascular tissue-engineered medicine products, 1524–1525
    decellularized extracellular matrix, 1525
    extracellular matrix, 1525–1526
    perfusion decellularization, 1525
    synthetic scaffolds, 1524
  cartilage reconstruction, 981–982, 982*f*
  and cell seeding, 1360–1361
  for cells, 4
  corneal replacement tissue, 1138–1140
  cultured meat technology, 1372–1373, 1373*f*, 1378–1379
  decellularized, 24
  decellularized ECM-based, 8
  electrospun, 9–10
  fibroblast-laden poly-caprolactone-based, 184–185
  fibrous, 9
  magnetic, 18–19
  material degradation, 4
  porosity, 1055*f*
  porous 3D, 12
  skeletal tissue engineering, 1014–1016
  spinal cord injury (SCI), 1052
    biomaterials, 1054–1055
    porosity, 1055*f*
  tendon and ligament tissue engineering, 992–994
    composition/material selection, 993
    structure/architecture, 993–994
Scaffold sterilization methods, 1562
Scarless healing/regeneration, 1319–1320
Scarring alopecias, 1299
Scarring, wound repair, 1318–1320
  pathological scars, 1318–1319
  scarless healing/regeneration, 1319–1320
Scatter factor, 84
Scavenger receptor class B (SRB1), 525
Schlemm's canal, 1153
Schwann cells (SCs), 354, 1055
SCID. *See* Severe combined immunodeficiency
Scleroderma digital ulcers, 1342, 1347–1348
SCNT. *See* Somatic cell nuclear transfer
SCP. *See* Slow cortical potentials (SCP)
Screw-based dispensing systems, 1394
Seager Electroejaculator, 875
Sebaceous glands (SGs), 1295–1296, 1332
Secondary fracture healing, 922*f*
Secondary germ, 1291
Secondary myotubes, 1378
Secondary-staged tissue-engineered graft, 1209–1210
Second heart fields (FHF), 579–580
Secreted phospholipases A2 (sPLA2), 1462
Secretomes, 829
Secretory cells, 709–710
Seeded cells, 642
Segmental diaphyseal defects, regenerative repair of, 1513–1516
Self-destruct chimeric antigen receptor, 771–772
Self-driving chimeric antigen receptor, 771–772
Self-healing, 2–4
Self-inactivating (SIN) vectors, 528
Self-inflicted gunshot wound
  with avulsive injury, 1203*f*, 1204*f*
  maxillofacial skeletal structures, disruption and loss of, 1204*f*
Self-organizing tissue culture, 1372–1375
Self-reparative capacity of bone, 919–922, 922*f*
Semilunar heart valves, 635–636
Seminiferous tubules, 871
Semipermeable gels, 334
Semipermeable membrane, 1425–1426
Semi-porous membrane, 1447
Semisynthetic biomaterials, 112
Sendai virus, 447, 447*f*
Senescence, 1381
Sensors, integration of, 19
Sensory fibers, 137–138
Septum transversum mesenchyme (STM), 723–724
Serum-free culture medium, 1377, 1462, 1472, 1489–1490
Serum proteins, 284
Severe combined immunodeficiency (SCID), 372, 501, 565
Sex-determining region Y (SRY)-box 9 (Sox-9), 1295
Shared-control BMI, 1041
Shear force, 238
Shear stress, 1279
Shear-thinning phenomena, 996
Shear thinning property, 984–985
Short bowel syndrome (SBS), 709
Short hairpin RNA (shRNA), 519
Sickle cell anemia, 766–767
Sickle cell disease, 767–768
Signaling-based strategies, 1195–1196
Signaling molecules, periodontal tissue engineering and regeneration, 1227–1232
  bone morphogenetic proteins, 1228
  crucial delivery barriers to progress, 1230–1231, 1231*f*
  enamel matrix derivative, 1229–1230
  fibroblast growth factor-2, 1228–1229
  gene delivery, alternative to growth factor delivery, 1231–1232
  growth/differentiation factor-5, 1229
  platelet-derived growth factors, 1228
  platelet-rich plasma, 1229
  stem cell–derived exosomes, 1230
  types of signals, 1228–1230
Signaling mothers against decapentaplegic (SMAD) proteins, 1013
Signaling pathways, 85
  in intestinal epithelium, 712
Signal regulatory protein alpha (SIRPA), 601
Signal transduction, 73–74, 73*f*
  events during cell–extracellular matrix interactions, 104–110
Silicone membranes, 1338–1339
Silicone polymer coating, 626–627
Silk, 321
Silk fibroin, 993, 1015–1016, 1138
Simple limbal epithelial transplantation, 1118
Simulated microgravity (SMG) stimulations, 186
Single blastomere, 422–424, 423*f*, 428
Single-cell injection, drawback of, 1239
Single-cell omics methods, 47
Single-cell RNA, 1256–1257
Single-cell RNA-Seq (sc-RNA-Seq), 1118, 1261, 1293–1294
Single-cell transcriptome analysis, 917–918
Single-chain antibodies, 529
Single-chain variable fragment (scFv), 770–771, 777–778
Single-dose regimen, 1556–1557

Single organ-on-chip systems, 193−194
Single unit activity (SUA), 1037−1040
Sinoatrial node (SAN), 581−582
Sinusoidal vessels produce CXCL-12 (SDF-1), 757−758
siRNA. *See* Small interfering RNA
SIRPA. *See* Signal regulatory protein alpha
Six1/4, 807
Six2, 809−814
Skeletal muscle, 150, 237, 1407
  cells, 145−146
  development, 145
  differentiation and repair, satellite cells in, 149−150
  tissue engineering of, 1357−1359
    cells, 1357−1358
    gel and seeding, 1358−1359
Skeletal stem cell (SSC), 917−919, 1008, 1012, 1012*f*
Skeletal tissue engineering
  cellular therapy, 1010−1013
  critical-sized defects, 1010
  cytokines, 1013−1014
  distraction osteogenesis, 1008−1010
  scaffolds, 1014−1016
  tissue engineering in practice, 1016−1017
Skeletal tissues, mesenchymal stem cells (MSCs), 899−901
  meniscus, 900−901
  tendon/ligament repair, 899−900
Skeletogenesis, 1007−1008, 1011−1012
Skeletogenic differentiation of mesenchymal stem cells, 888
Skin, 1446−1447
  as active immune organ, cutaneous epithelial stem cells, 1300−1301
    cross talk between hair follicles and immune system, 1300−1301
    inflammatory memory of skin cells, 1301
  bioprinting, 1408−1409
  functions of, 1309
  structure and function, 1331
  tissue engineering systems, monitoring and real-time control of, 1464
Skin autograft harvesting without scarring, 1322
Skin cells
  inflammatory memory of, 1301
  wound repair, tissue engineered therapy, 1320−1322
    cellular and acellular, 1320
    engineered dermal constructs, 1321
    engineered epidermal constructs, 1320−1321
    engineered skin substitutes, 1321−1322
    skin autograft harvesting without scarring, 1322
Skin homeostasis, 1297, 1301
Skin loss, 1309
Skin-penetrating nanoparticles, 537
Skin renewal, epidermal proliferative unit *vs.* committed progenitor, 1290−1291
Skints, 1299
Slicing, 1391−1392
Slit guidance ligand 3 (SLIT-3), 897
Slow cortical potentials (SCP), 1039
Slow-cycling stem cell pool, 1290−1291
Slow turning lateral vessel (STLV), 471
SLRPs. *See* Small leucine-rich repeat proteoglycans
Small interfering RNA (siRNA), 519−521, 520*f*, 521*f*, 528, 530*f*, 531, 955
Small intestinal epithelium, 711*f*
Small intestinal submucosa (SIS), 641, 851−852, 864, 943−944, 998−999
Small intestinal villus, 710*f*
Small leucine-rich repeat proteoglycans (SLRPs), 121−122, 128
Small molecule-induced differentiation, 1580
Small-scale production methods, 1382
Smart biomaterials, 2−4, 21−22
  with 3D bioprinting, 4
  applications of, 3*f*
SMG. *See* Submandibular salivary gland
Smooth muscle cells (SMC), 459, 621, 846, 849, 1399−1400
Snai2, 814
Snail-1 misexpression, 82
Social Security, 1574
Sod 2 knockdown mouse model, 1125
Sodium alginate, 1408
Sodium dodecyl sulfate (SDS), 833, 864
Sodium hyaluronate microparticles, 1059−1065
Soft and hard tissue defects
  nonvascularized grafts, 1205−1207
  vascularized grafts, 1205
Soft lithographic micropatterning, 1403
Soft tissue pedicled flaps, 1205
Solid freeform fabrication, 1512
Solid scaffolds, 1234, 1501
SolidWorks, 295
Somatic cell nuclear transfer (SCNT), 423−424, 457−458
Somatic cell nucleus, 481*f*
Somatic cells, into induced pluripotent stem cells, 445−446
Sonic hedgehog (SHH), 693−694, 1254−1255, 1295−1296, 1298
SOX2, 1254−1255
Sox5, 894
Sox6, 894
Sox9, 813, 894, 954, 1254−1255, 1463
SPARC, 210−212
Spatial tissue inhomogeneity, 42−43
Special Protocol Assessment (SPA), 1561
S1P1-endothelial differentiation sphingolipid G-protein-coupled receptor-1 (EDG1), 1280
Spermatogenesis, 873
Spermatogonia, 871−872
Spermatogonial cells, 872*f*
Spermatogonial stem cells (SSCs), 871−873
Sperm banking, 871−872
Sperm cryopreservation, 871−872
Spheroidal models, 1538−1540
Spheroidal organoid fabrication methodologies, 1539*f*
Spheroids, 13−19, 1425−1427, 1432−1434, 1447, 1538−1539
Spherox, 1504
Sphingosine-1-phosphate, 1377
Sphingosine-1-phosphate (S1P), 1262
Spina bifida, 461
Spinal cord injury (SCI), 875, 876*f*, 1048−1049, 1426−1427
  animal models of, 1052−1054, 1054*t*
  available clinical interventions, 1049
  bioengineering for integrated spinal cord biocompatibility, 1052, 1053*t*
  biomaterial
    fabrication for repair, 1054−1058
    natural polymers, 1058−1072, 1060*t*
  clinical translation, promise of, 1080
  continuum of, 1050*f*
  epidemiology, 1047
  injury mechanism classifications, 1048
  natural and synthetic polymer scaffolds, 1047
  physical, cellular and molecular barriers, spinal cord regeneration, 1049−1051
  role of tissue engineering in, 1051−1052
  scaffold porosity, 1055*f*
  spinal cord organization, 1047−1048, 1048*f*
Spinal ejaculation generator, 875
Spinal generator of ejaculation (SGE), 875
SPION. *See* Superparamagnetic nanoparticles
Spiral ganglion neurons (SGN), 1098
Split chimerism, 372
Split-thickness autografts, 1321
Split-thickness skin grafts, 1322
Spontaneous epidermal hyperplasia, 1295
Sprague Dawley rat uterine segments, 864
Spry1, 808−809
Squamous cell carcinoma
  ablative defect of, 1205*f*
  floor of mouth and tongue, 1203*f*
SSc. *See* Systemic sclerosis
S-shaped body (SSB), 810−811
S-100 staining, 856
Stabilized mRNA, 1012−1013
Staged maxillary sinus floor augmentation, 1209
Starch, 323−324
Stargardt macular dystrophy, 1163−1164
Stargardt's disease, 1578−1579
Stargardt's macular dystrophy, 1122
STAT3, 1299
STAT-dependent pathways, 1097
Stationary wavelet transformation, 1178
21st Century Cures Act, 1473, 1553, 1563−1564
Steady-state visual evoked potential (SSVEP), 1038−1039
Stem cell, 710−712
  for erectile dysfunction, 876−877
  glucose-responsive, 666−667
  interactions, 38
  pluripotent, 717
  populations, variability, 40−41
Stem cell-based therapeutic development, 438−440

Stem cell-based tissue engineering of teeth, 1189
Stem cell-derived cardiomyocytes, purification and scalable production of, 601
Stem cell differentiation
  gene transfer to instruct, 508–509
  high-throughput assays for directing, 475–477
  magnetic particles in, 478
Stem cell dynamics, modeling, 37–41
Stem cell factor (SCF), 767, 1262
Stem cell fate, 38, 40
Stem-cell growth, gene transfer to control uncontrolled, 508
Stem cell niche, 711*f*, 758
Stem cell populations, 260
Stem cells, 5, 22, 37, 145, 148, 150–151, 407, 457–459, 709, 846, 946–947, 1212–1213, 1241, 1347–1348
  in alopecia, 1299–1300
  amniotic fluid (AF), 458–459
  application of, 480
  cardiac differentiation of human, 599–601
  cardiac resident mesenchymal, 584–585
  definition of, 1115
  derivation of, 468*f*
  diabetic retinopathy, 1155–1156
  embryonic, 188
  in eye
    endogenous ocular stem cells, 1115–1121
    nonocular stem cells, 1121–1126
    ocular stem cells, 1116*f*
  fate, 38
  fetal, 457
  fetal liver, 724
  gene transfer to, 508
  glaucoma, 1154–1155
  of intestine, 709–714
  intestine with, 714–717
  lines from farm-animal species, 1376–1377
  mesenchymal, 643–644
  modes of action assigned to, 848*t*
  neonatal, 457
  in ocular repair and tissue engineering, 1126
  perinatal, 458–459
  pluripotent, 458, 586–588
  potential of, 508
  safe media for, 1377
  sources, 5, 867
  therapeutic applications of, 469
  tissue-derived, 421
  wound repair, 1322–1324
Stem cells factor (SCF), 757
Stem cells from apical papilla (SCAP), 1222–1225, 1227*t*
Stem cells from exfoliated deciduous teeth (SHED), 1222–1223
StemCells Inc., 1028
Stem cells in kidney development and regeneration
  kidney development, 805–813
    early embryonic origins of nephrogenic tissues, 806–808, 806*f*
    nephric duct and ureteric bud, 808–809
    nephron endowment, 812–813
    progenitor cell, maintenance and differentiation of, 809–811
    stromal lineages in kidney organogenesis, 811–812
  kidney repair and regeneration, 813–817
    renal tissue from pluripotent stem cells (organoids), 815–817
    sources of nephrogenic cells, 814–815
    stem cells in kidney repair, 813–814
Stem-cell strategies, gene transfer with, 508–509
Stem cell therapy, 586, 927, 1473–1474, 1476
Stemness, 1291
Stem/progenitor cells
  derived from kidneys
    renal stem cells in Bowman's capsule, 828
    renal stem cells in papilla, 828
    renal stem cells in tubules, 828–829
  in kidney regenerative biology, 817*f*
  intestine with, 714–717
Stenosis, aortic, 637
Stereolithography (SLA), 305–306, 996–997, 997*f*, 1395, 1397
  components of, 1395
  digital light processing (DLP), 1395–1397
Stereolithography (SLA)-based printing, 302–303
Stereotactic method (stereotaxis), 1032
Steroid receptor systems, 507
Steroids, 770–771
Stevens–Johnson syndrome, 1137
Stimulation, electrical, 348–349
Stimuli, 407
Stirred tank bioreactors, 1470
STM. *See* Septum transversum mesenchyme
Stochasticity, 39*f*, 40–41
StrataGraft, 1322, 1339–1340
Strategic ignorance, 1364
Stress fibers, 239
Stress-related mitochondrial pathways, 1094
Stretch-activated ion channels, 239–240
Stroke, 1029
Stroma, 1138–1139
Stromal cell–derived factor-1 (SDF-1), 835, 836*t*, 837, 899–900, 919–921, 1299
Stromal cells, 557–558, 1257
Stromal progenitor (SP), 809, 811–812
Stromal stem cells, 1119
Stroma tumor, 1428
Stromelysin (MMP-3), 1311–1312, 1314
Strong shear-thinning behavior, 1396–1397
STRO-1-positive dental follicular progenitors, 1224–1225
STRO-1 positive mesenchymal stem/progenitor cells, 887
Structure-based 3D bioprinting, 1391
  biodegradable synthetic polymers, 1399
Styrene, polymerization of, 284
SUA. *See* Single unit activity (SUA)
Subchondral bone
  penetration of, 972–973
  reconstruction, 899
Subcutaneous fat, 1485
Subcutaneous implanted scaffolds, 928*f*
Subcutaneous (SC) space, 408–409
Submandibular salivary gland (SMG), 220
Substrate-mediated delivery, 540
Subtotal cystectomy, 856
Subtotal cystoplasty, 853*t*
Suicide gene transfer strategies, 508
Sulfated alginate, 984–985, 984*t*
Superoxide, 787–788
Superoxide dismutase (SOD), 787–788
Superparamagnetic nanoparticles (SPION), 19, 1139–1140
Supervised machine learning methods, 46
Supplementary motor area (SMA), 1039
Support vector machines (SVMs), 46
Suprapubic catheterization, 855
Surface adsorption, of homopolymers, 284
Surface chemistry, 349–351, 1212
  of polymers, 281
Surface conjugated hemoglobin, 790–791
Surface markers, mesenchymal stem cells, 884–885
Surface modification techniques, 281–283, 351
Surface morphology, on cell behavior, 284–285
Surface topography, 351–352
Suspension, cell interactions with polymers in, 286
Suspension culture techniques, 286
Sustainable bioreactors, 188–189
SVMs. *See* Support vector machines
Sweat glands, 1296
Syndecan-4, 107, 209
Syndecans, 94, 128
Syndromic hereditary hearing loss, 1093
SynerGraft valve, 642, 647
Synovium-derived MSCs, 886
Synthetically derived biomaterials, 1422–1424
Synthetic and coral-derived porous HA, 1235
Synthetic and natural polymeric scaffolds, 943
Synthetic AV grafts, 619
Synthetic biology advance, 1477
Synthetic bone, 930
Synthetic cationic glycopolymers, 536
Synthetic derived polymers, 333
Synthetic grafts, 622–623, 998–999
Synthetic hydrogel, 984–985
Synthetic implants, 559
Synthetic materials, 351–352, 459, 561–562
Synthetic PEG hydrogels, 896
Synthetic peptides, 924*t*
Synthetic perfluorocarbons (PFCs), 785
Synthetic photoreceptors, 1174
Synthetic polymers, 186, 275, 276*t*, 278, 280–283, 297, 298*t*, 326–333, 531–534, 640–641, 670, 831*t*, 857, 864, 1138, 1208–1209, 1502
  with adsorbed proteins, 282–283
  advantages, 1234
  biomaterial, 1072–1080, 1074*t*
    nonbiodegradable hydrogels, 1077–1080
    poly α-hydroxy acid polymers, 1073–1077
  hydrolytically degradable polymers, 1234

poly(L-lactic acid) (PLA), 1234–1235
scaffolds, 1015
Synthetic proteins, 284
Synthetic scaffolds, 186, 715–716, 850–851, 851*t*
biodegradable properties, 850–851, 851*t*
porosity, 851
Synthetic/semisynthetic biomaterials, 1360–1361
Systemic delivery systems, 521
Systemic sclerosis (SSc), 210
Systemic sclerosis (scleroderma) digital ulcer, 1343*f*
Systems biology approach, 1362

T

Tamoxifen-inducible lineage tracing of K141 nail basal epidermal cells, 1297
tanCAR, 771–772
Target cells, chromosomal DNA of, 495
Targeted stem cell therapy, 1196
T-box transcription factor TBX1, 689, 693
Tbx1, 689
T cell, 365, 770–773
development, 681–682
differentiation, in vitro, 683–685
T-cell receptor (TCR), 770–771
T cells redirected for universal cytokine killing (TRUCKs), 771–772
Tears and osteochondral fractures, 970
TE bioreactor systems, 1443
Tegafur, 1448–1451
TEI. *See* Tissue-engineered intestine
Telomerase activity (TA), 846
Temperature-responsive culture system, 865
Temperature, tissue engineering systems, 1461
Temporalis muscle/temporoparietal flap, 1207–1208
Temporomandibular defect repair, bioprinting, 985
Temporomandibular joint (TMJ), 1212–1214
condyle, 1215
degenerative diseases of, 1202–1203
Tenascin, 126–127, 1311
Tenascin-C, 208–209, 772
Tendon, 1407–1408
Tendon and ligament tissue engineering, 989–990
bioink, ligament and tendon structures, 997–998, 998*f*
composition, 990
function, 990–991, 991*f*
requirements for, 991–996, 992*f*
bioactive factors, 995–996, 995*f*
cell, 994–995, 994*f*
scaffold, 992–994
structure, 990
three-dimensional bioprinting and bioink, 996–997
bioink's shear thinning and viscosity, 996*f*
clinical application, 998–999
G-code, 996
hydrogel scaffold, 996*f*
inkjet printed graphene oxide–gelatin pattern, 997*f*
SLA printed high-resolution microneedles, 997*f*
Tendon autograft, 998–999
Tendon/ligament repair, 899–900
Tendon tissue architecture, 900*f*
Tenocytes, 1407–1408
Tenogenic/fibroblastic pathway, 994
Tense (T) quaternary state, 785–787, 787*f*
Tensile stress, 103–104
Tension, 237
cyclic patterns of, 250
sensing at adherens junctions, 241*f*
sensing at focal adhesions, 242*f*
TEPC. *See* Thymic epithelial progenitor cell
Teratocarcinoma–derived cell line in stroke, 1029
Teratoma formation, 1013
Tertiary dentin, 1194
Tertiary dentinogenesis, 1192–1193
Terumo Quantum, 1476
Testes, 871–874
androgen-replacement therapy, 873–874
spermatogonial stem cell technology, 871–873
Testicular dysfunction, 873–874
Testicular tissue grafting, 873
Testosterone, 873–874
Tetracycline response element (TRE), 505–506
Tetracycline-responsive system, 505–506
Tetracycline transactivator, 505–506
Texture of meat, 1363
Therapeutic agents, 563
Therapeutic angiogenesis, 266
Therapeutic cells, 405–407
Therapeutic cloning, 481*f*
Therapeutic proteins, 403, 405
Therapeutic scaffolds, 563
Therapeutic trans-splicing, 507
Thermal inkjet bioprinters, 1392–1394, 1418
Thermal liftoff, 1379
Thermal plastic polymers, 997–998
Thermogelation, 1209
Thermoresponsive polymer, 1067–1069
Thomson Cocktail, 1121
Thoracic thymus, 688
Three-dimensional bioprinted implantable tissue constructs, 1403–1409
Three-dimensional bioprinted vascular structures, 1400, 1401*f*
Three-dimensional bioprinting, 2, 15–16, 19–20, 832, 926–927, 982, 996–997, 1391, 1418, 1419*t*, 1420*f*, 1430, 1512
advances in, 25
applications of, 1402*t*
bioink's shear thinning and viscosity, 996*f*
biomaterials as bioinks
biodegradable synthetic polymers for structure-based 3D bioprinting, 1399
hydrogel-based bioinks for cell-based bioprinting, 1396–1399
scaffold-free cell printing, 1399–1400
bone and cartilage reconstruction, 982, 983*f*
of cancer models, 1428
clinical application, 998–999
dispenser system, 1392
extrusion, 12–13
extrusion-based bioprinting, 1394, 1404*t*
advantage of, 1394
G-code, 996
hybrid 3D bioprinting technology, 1396
hydrogel scaffold, 996*f*
inkjet, 12
inkjet printed graphene oxide–gelatin pattern, 997*f*
jetting-based bioprinting, 1392–1394, 1403, 1404*t*
advantages, 1394
drawbacks of, 1394
laser-assisted bioprinting, 1394–1395
laser-based stereolithography, 1395
medical image to printed bioengineered tissue, 1391–1392, 1392*f*
osteochondral constructs, 985
polymeric hydrogels for, 13
printing cartridges, 1392
regulatory process, market, 1563
SLA printed high-resolution microneedles, 997*f*
smart biomaterials with, 4
three-axis stage, 1392
in tissue engineering applications, 1400–1409
three-dimensional bioprinted implantable tissue constructs, 1403–1409
three-dimensional bioprinted vascular structures, 1400, 1401*f*
in vitro tissue models, 1400–1403, 1402*t*
for tissue regeneration applications, 1404*t*
of vascular networks, 25
3D bioprinting brain tissue, 1425–1426
Three-dimensional (3D) cell culture techniques, 14, 179, 1444–1445, 1537
3D collagen scaffold, 831–832
Three-dimensional (3D) fibrillary protein network, 1051
Three-dimensional (3D) imaging techniques, 1204
Three-dimensional (3D) implants, 158
Three-dimensional liver constructs, 741–742
3D liver microtissue organoids, 1543–1544
3D liver organoids and expansion, 727–730
Three-dimensional organ bioprinting, 138
3D organoid-based cultures, 1151–1152
3D patient-specific models, 1454–1455
3D polyvinyl formal resin scaffolds, 760
3D printed kagome-structure scaffold, 927
3D printed polylactide-*co*-glycolide/tricalcium phosphate composite scaffold, 929
3D proximal tubules-on-a-chip, 832
Three-dimensional (3D) printing (3DP) technology, 295, 924, 1138, 1301–1302, 1417, 1447, 1472
bone tissue engineering, 1513–1516
used in bone biofabrication, 925*t*
3D RNA delivery technique, 472

Three-dimensional scaffolds (3D scaffolds), 343
  cell transport and vascularization, 345
  design and engineering, 343–355
  mass transport, 344–346
  mechanics, 346–348
  pore architectures, 344–346
Three-dimensional (3D) skin organoid cultures, 1301
3D testicular organoid model, 1542
Three-dimensional tissue growth in vitro, 43–44
3D tissue models, 2, 1417–1418
Three-joint complex, 937
Thrombocytopenias, 779
Thromboembolism, 635
Thrombomodulin, 1277–1278
Thrombopoietin (TPO), 757
Thromboresistance, 627
Thrombosis, 619, 1525
Thrombospondin (TSP), 126
  thrombospondin-1, 205–207
    antiangiogenic properties, 205–206
    CD-36, 205–206
    CD-47, 206
    TGF, 206
  thrombospondin-2, 207–208
Thrombospondin-1 (TSP1), 1262
Thrombospondin 4, 101
Thymic dendritic cells, 681
Thymic epithelial cell, 682–685
  differentiation, regulation of, 695
  maintenance and regeneration of, 696–697
  origin of, 686
Thymic epithelial progenitor cells (TEPC), 686–687, 695–697
Thymic epithelium compartment, complexity of, 682–683
Thymic stroma, 681
Thymocyte progenitors, double negative (DN), 681–682
Thymocytes, 682*f*
Thymus
  cervical thymus in mouse and human, 688–689
  development, 688
  functional diversity, 683
  histology of postnatal, 682*f*
  reconstitution, strategies, 697–698
  structure and morphology of, 681–682
  thoracic, 688
Thymus organogenesis, 685–689
  cellular regulation of, 685–686
  molecular regulation of, 689–696, 690*f*
  specification, 692–695
Thyroid transcription factor-1 (TTF-1), 1254–1255
Time-controlled GFP expression, 1118
Timolol, 1153
Timothy syndrome (TS), 449
Tisagenlecleucel, 770–771
Tissue, 77
  biodistribution/targeting, 521–523
  as integrated systems in body, 136–138
  remodeling, 133
Tissue culture bioreactors, 166
Tissue culture cells, 68
Tissue-culture PS (TCPS), 280–281
Tissue-derived matrices, 850–851
Tissue development and homeostasis, 119
Tissue-engineered and regenerative medicine products, 1562–1563, 1562*f*
  safety and effectiveness, for cell-scaffold, 1562*f*
  3D bioprinting, 1563
Tissue-engineered cartilage, optimal maturation of, 161–162
Tissue-engineered cartilage constructs, 159
Tissue-engineered cellular three-dimensional renal constructs, 830–835
  decellularization/recellularization strategy, 832–835
  natural and synthetic polymers, 830–832
Tissue-engineered drug screening platform, 160–161
Tissue engineered heart valve (TEHV), 636, 640–643, 645–647
  clinical applications of, 647–648
  neotissue development in, 645–646
Tissue-engineered intestine (TEI), 709
  generation of, 718
Tissue-engineered Medical Product (TEMPs), 1522*f*
Tissue-engineered myocardium, 1356–1357
Tissue engineered products, polymers to create, 333–334
Tissue-engineered skin products, 1483–1484
  clinical trials, 1492–1493
  commercial production of, 1487
    allogeneic cell source, 1488
    Dermagraft, 1487–1488
    final sterile fill, avoidance of, 1488
    product development, 1487
    regulation, 1487
    shelf life, 1488–1489
    size, user convenience, 1489
    TransCyte, 1488
    viability of product, 1488
  commercial success of, 1494–1495
    mechanism of action, 1494–1495
  components of, 1484–1489
    adnexal structures, 1487
    extracellular matrix, 1485, 1486*t*
    fibroblasts, 1485
    immune system, 1486
    keratinocytes, 1485
    melanocytes, 1486–1487
    scaffold, 1484
    subcutaneous fat, 1485
  Dermagraft and TransCyte
    manufacture of, 1489–1490, 1489*f*
    production processes, 1490–1492
  immunological properties of, 1493–1494
  types of, 1484
Tissue-engineered small intestine (TESI), 712, 714, 715*f*, 716–717
Tissue-engineered vascular grafts (TEVG), 1525–1526
  in vitro, 623
  in vivo, 625
Tissue engineering
  aspects of, 2*f*
  challenges, 19–21, 26
  current state of, 53–54
  gene delivery and, 542*f*
  machine learning in, 45–47
    regulatory network inference, 47
    supervised method, 46
    unsupervised method, 46–47
  morphogens as signaling cues in, 140
  regulatory considerations for, 58–60
  relevance for, 111–113
Tissue-engineering-based therapies, 467–468
Tissue engineering/regenerative medicine (TE/RM), 363
Tissue engineering scaffolds
  electrical conductivity, 348–349
  mechanical properties of, 346–347
  spatial control, 354–355
  surface properties, 349–352
    surface chemistry, 349–351
    surface topography, 351–352
  temporal control
    scaffold degradation, 352–353
    soluble bioactive factor delivery, 353–354
Tissue engineering systems (TESs), monitoring and real-time control of
  amino acids, 1462
  carbon dioxide, 1461
  cell-level monitoring, 1463
    reporter-based gene expression imaging, 1463, 1463*f*
  challenge in, 1460
  definition of, 1459
  flowchart for, 1459, 1460*f*
  glucose, 1461–1462
  lactate, 1462
  levels of, 1459, 1460*f*
  mechanical properties, 1462–1463
    optical coherence tomography (OCT), 1463
    ultrasound elastography, 1462–1463
  nondestructive technologies, 1459
  oxygen, 1461
  pH, 1461
  proteins, uptake and secretion of, 1462
  temperature, 1461
  tissue-level monitoring, 1462
    extracellular matrix amount/levels, 1462
    extracellular matrix structure, 1462
  tissue-specific considerations, 1463–1464
    cartilage monitoring and real-time control, 1463–1464, 1464*f*
    skin, 1464
    tissue type and monitored/controlled variables, 1465*t*
Tissue engineering theory, 640
Tissue extravasation, 788
Tissue flaps, 559
Tissue formation, 37, 347
Tissue glues, 2–4
Tissue graft, 1526

Tissue growth
on complex surfaces in vitro, 42–43
development, 41–45
in monolayer and 3D culture, 41*f*
Tissue inhibitor of matrix metalloproteases 2 (TIMP-2), 1319
Tissue inhibitor of metalloproteinase (TIMP), 636–637, 1317, 1463
Tissue inhibitor of metalloproteinase-1 (TIMP-1), 954, 1319
Tissue maintenance, environmental conditions for, 112–113
Tissue morphogenesis, 133, 247–248
biology of, 133–138
cell–cell interactions during, 136
extracellular matrix as a key regulator of, 135–136
mechanical interactions during, 247*f*
morphogens as bioactive signaling molecules during, 134–135
and regeneration, 138*f*
Tissue neovascularization, 17–18, 25
Tissue organoid models and applications, 1537
applications, 1542–1545
developmental biology, 1544–1545
drug analysis, 1543–1544, 1543*f*
organ-on-a-chip (OOC), 1544, 1544*f*
tumor and disease models, 1542–1543
brain organoid, 1540–1541, 1541*f*
cardiac organoid, 1539–1540
cell sources, 1537–1538
limitation of, 1538
gastrointestinal tract organoid, 1541–1542, 1542*f*
liver organoid, 1540
lung organoid, 1541
pancreatic organoid cultures, 1542
3D testicular organoid model, 1542
types of, 1538–1539
Tissue remodelling, in healthy and diseased environments, 140–141
Tissue-replacement therapies, 1589
Tissue response, 317, 334
Tissue-restricted antigens (TRAs), 683
Tissue-specific promoters, 507
Titanium mesh scaffold, 1516–1517
Titanium oxide nanoparticles, 18
Titin mutation-related dilated cardiomyopathy, 611
Title 21 of the Code of Federal Regulations (CFR) Part 1271, 1553
TKKTLRT, 852–854
TLRs. *See* Toll-like receptors
T lymphocytes, 65–66, 681
TMC. *See* Trimethylene carbonate
TNF. *See* Tumor necrosis factor
TNFR1 expression, 955
TNF stimulated gene 6 (TSG6), 890
Tobramycin, 1094–1095
Toll-like receptor 3, 1298
Toll-like receptor-7 ligand, 1301
Toll like receptors (TLRs), 95, 670, 788
Tool path generation, 1391–1392
Tooth development, 1187–1189, 1188*f*
Top-down approach, 924, 996, 1395–1396
Total joint reconstruction, 1202–1203
Totally implantable cochlear implant (TIKI), 1101
Toxicity, screening for, 437
Trabecular bone-derived MSCs, 886
Trabecular meshwork (TM), 1153
Trabeculectomy surgery, 1153
Tracheal allotransplantation, 1266
Tracheospheroid models, 1541
TRAFFIC (thread-reinforced alginate fiber for islets encapsulation) device, 408
Transamniotic stem cell injection, 461
Transcatheter deployment carries, 640
Transcatheter valve replacement, 640
Transcriptional regulators, 145
Transcription factors, 67, 530, 658–659, 660*f*, 1293
basic helix-loop-helix (bHLH) family of, 147
muscle-specific, 147
regulation of epithelial–mesenchymal transition, 82
Transcriptome profiling, 1301
Transcutaneous oxygen tension ($TcPO_2$), 1334–1335
Transcutaneous photopolymerization, 1209
TransCyte, 1321, 1339, 1483–1484, 1488–1489, 1494–1495
design concepts for, 1487, 1488*t*
manufacture of, 1489–1490, 1489*f*
bioreactor design, 1490
cells, 1489
medium, 1489–1490
production processes, 1490–1492
distribution and cryopreservation, 1491–1492
problems with commercial culture, 1492
release specifications, 1491
Transcytosis, 523–524
Transdifferentiation, induced, 448
Transduction, signal, 73–74, 73*f*
Transepithelial electrical resistance sensors, 1449
Transferred gene
chromosomal *vs.* extrachromosomal placement of, 495
regulated expression of, 505–507
regulation of expression of, 506*t*
Transfer vectors, gene, 495–502
Transforming growth factor-α (TGF-α), 1312, 1315
Transforming growth factor-β (TGF-β), 206, 836*t*, 888, 894, 949*t*, 952–953, 1116–1117, 1192–1193, 1195, 1209, 1228, 1312–1317, 1333, 1335
Transforming growth factor β-1 (TGF-β1), 953, 980, 1008, 1213, 1215, 1541
Transforming growth factor (TGF) β3, 947
Transforming growth factorβ-binding proteoglycans, 99
Transgenes, multiple, 164–165
Transglutaminase, 207
Transient amplifying cells (TACs), 1115–1117
Transit-amplifying progeny, 1290–1291
Transit amplifying (TA) zone, 710
Transmembrane proteins, 80
Transplantation
site, 408–409
in utero, 371–376
barriers, 373–376
clinical experience, 376
murine experiments, 372
Transplanted cell rejection, 404
Transplanted cells, 740
and materials, rejection and protection of, 403–404
Transplanted hepatocytes, 743
Transplanted HIOs (tHIO), 717
Transplant niche, immune cell behavior in, 412
Transport tubes, 1174
Trans-splicing gene transfer strategies, 507
Transverse rectus abdominis myocutaneous (TRAM) flap, 559
Trap hypothesis, 1334
TRAs. *See* Tissue-restricted antigens
Traumatic cervical spinal cord injury, 1049*f*
Travoprost, 1153
TRE. *See* Tetracycline response element
Tregs, 770–772, 849–850, 889, 1124, 1300
Triblock methacrylated polyHPMA-lac-PEG copolymer, 984–985
Tricalcium phosphate, 1015
Trimethylene carbonate (TMC), 317
Triple helices collagens, fibril-associated collagens with interrupted, 122–123
Triple helix formation, 121
Tristable system, 38–40
Tritiated thymidine, 1292–1293
Triton-X, 864, 1275
Triton X-100, 833
Troglitazone (Rezulin), 1454
Trophic factors, 829
Tropism, of gene transfer vectors, 504*f*
Trovafloxacin, 1543–1544
Trovafloxacin dose-dependent toxicity, 1430–1431
TS. *See* Timothy syndrome
Ttissue-derived organoids, 815
T-type channel blockers, 1097
Tubules, renal stem cells, 828–829
Tubulogenesis, 811
Tuft cells, 709–710
Tumor and disease models, 1542–1543
Tumorigenic transformation of MSCs, 891
Tumor metastasis, 1427–1428
Tumor metastasis-on-a-chip platforms, 1450
Tumor microenvironment (TME), 1427–1428
Tumor necrosis factor (TNF), 100
Tumor necrosis factor alpha (TNF-α), 890, 919
Tumor organoid models, 1543
Tumors, 248
Tunneling nanotubes (TNTs), 586
Turbulent flow, 192
Two-dimensional (2D) cell culture, 760, 830, 1301, 1400–1403, 1417, 1443–1445, 1537, 1543
Two-dimensional liver culture, 741
Two-dimensional (2D) tissue culture, 866

Two- or three-walled intrabony defects, 1235
Two-photon polymerization (TPP)–based bioprinting, 1419*t*, 1421
Two-photon polymerized acrylated poly (caprolactone) (PCL) scaffolds, 1123
Type I collagen, 892, 938–942, 980, 990–993, 1224–1225, 1311, 1316, 1403, 1404*t*, 1484, 1501–1502, 1501*t*
Type I cytokines, 1116–1117
Type I diabetic (T1D) patients, 665
Type II collagen, 938–943, 948, 953–954, 971, 979–980, 982, 1406, 1501–1502
Type II cytokines, 1116–1117
Type III collagen, 952, 1319–1320, 1501–1502, 1501*t*
Type III cytokines, 1116–1117
Type VII collagen fibrils, 1312
Type XVII collagen (COL17A1/BP180), 1298–1299

U

UB-derived Wnt9b, 810–811
Ultrasound elastography, 1462–1463
Ultroser G, 1377
Umbilical cord, cells from, 458
Umbilical cord blood (UCB), 757
  UCB-derived cells, 458
  UCB–derived MSC, 886
Umbilical cord cells, 643–644
Umbilical vein EC (UVEC), 876–877
Unit processing controls, 1469
Unsupervised machine learning methods, 46–47
uPA. *See* Urokinase plasminogen activator
Upper hair follicle, 1295
Upper jaw, defects of, 1207–1208
Ureteric bud (UB), 806, 808–809, 811–812
Urinary bladder, 845
Urinary diversion, 854–855
Urine-derived stem cells (USCs), 848–850
Urokinase plasminogen activator (uPA), 101
Urokinase-type plasminogen activator antigen, 1278
Urothelial cell (UC), 846
Urothelial-specific cell markers, 849
Urothelial tissue, 845
Urothelium, 845
US Center for Medicare and Medicaid Services, 1337
US Department of Agriculture (USDA), 1381–1382
US FDA forms, 1557–1558
US Food and Drug Administration (FDA), 850, 894, 1025, 1167, 1381–1382, 1553–1554
  international regulatory activities, 1568
  sponsor meetings, 1557
US Food and Drug Administration forms, 1557–1558
Uterine cervix tissue engineering, 865
Uterine infertility, 863
Uterus, female reproductive organs, 863
  acellular tissue engineering, 864
  cell-seeded scaffolds, 864–865
  scaffold-free approaches for, 865
UV-compatible rheometer, 300

V

Vaccine manufacturing, 1470
Vaginal agenesis, 866
Vaginoplasty techniques, 866
Valproic acid, 1449
Valves, 1526–1527
  current valve prostheses, 1526
  tissue-engineered valves, 1527
Valvular endothelial cells (VECs), 636
Valvular heart disease (VHD), 1428–1429
Valvular interstitial cells (VICs), 636–638
Valvular neotissue, 644
Vancomycin-eluting PMMA cement beads, 1514
Various epidermal growth factors (VEGFs), 1116–1117
Vascular access, hemodialysis, 619–620
Vascular bone, development of, 925–926
Vascular-casting approach, 1400
Vascular cell adhesion molecule 1 (VCAM 1), 497
Vascular dysregulation, 1153–1154
Vascular endothelial cell growth factor (VEGF), 1312–1313
Vascular endothelial growth factor (VEGF), 94, 97–98, 103, 108, 136, 140, 266, 509, 776, 836*t*, 852–854, 866, 888, 901, 923*t*, 925–926, 1014, 1155, 1262, 1280, 1315–1316, 1324, 1429–1430, 1487
  isoforms, 1315
  VEGF-A, 1315
Vascular endothelial growth factor-A (VEGF-A), 1014
Vascular endothelial growth factor (VEGF)-dependent ossification, 1011–1012
Vascular endothelial growth factor (VEGF) signaling, 85
Vascular endothelial progenitor cells (EPCs), 926
Vascular grafts, 1525–1526
  autologous saphenous veins, 1525
  scaffold-based TEVGs, 1526
  scaffold-free grafts, 1526
  in vitro tissue-engineered, 623
Vascular interventions, 619*f*
  inflammatory response to, 620
Vascularization, 661, 832, 1400, 1528
  in bioengineered tissue, 2
  cell transport and, 345
  control of, 1335–1336
Vascularized flaps, 1207–1208
Vascularized free flap, 1209–1210
Vascularized grafts, soft and hard tissue defects, 1205–1206
Vascular mesenchymal cells (VMCs), 44–45
Vascular pathology, in peripheral arterial disease (PAD), 621*f*
Vascular progenitor cells, 473
Vascular repair, 1262
Vascular smooth muscle cells (VSMCs), 98–99, 617
Vascular tissue engineering, 623–630, 746–747
  developmental biology cues in, 618
  host integration, 747
Vascular tissue models, 1432–1435, 1433*f*
Vasculature, 251, 265–266, 760
  in cardiac patch, 608
  innervation and, 637
Vasculitis, treatment of, 1344*f*
Vasculogenesis, 16, 1258
Vas deferens, engineering, 874
Vasoactive intestinal peptide (VIP) signaling, 224
Vasoconstriction, 788, 790, 1096
Vaso vasorum, 617, 626, 630
V-cadherins, 1312
VCAM 1. *See* Vascular cell adhesion molecule 1
VE-cadherin *(Cdh5)*, 849, 1258
VE-Cadherin-based barrier function, 1278
VECs. *See* Valvular endothelial cells
Vectors
  adeno-associated virus (AAV), 499–500
  adenoviral, 527–528
  gene transfer, 495–502
  lentivirus, 501–502
  nonviral, 497
  viral, 519, 527–528
  viral gene transfer, 502
Venous, reconstruction, 619
Venous insufficiency, 1341–1342
Venous leg ulcers (VLUs), 1317–1318, 1321–1322, 1340–1341
Venous thromboembolism, 1434–1435
Venous ulcers, 1309
Ventricular assist devices (VADs), 594
Ventricular cardiomyocyte progenitors, 581
Verapamil, 1097
Vesicular glutamate 3 (VGLUT3), 1099
Vesicular stomatitis virus (VSV), 501
Vessel-on-a-chip, 1447–1448
Vessel wall, normal and pathologic composition of, 617–618
VICs. *See* Valvular interstitial cells
Vimentin, 239
Viral delivery, to engineer tissues, 538–539
Viral gene therapy, 526–527, 954–955
Viral gene transfer vectors, 502, 503*t*, 1210–1211
Viral nucleic acid delivery, 526–530
Viral particles, 529
Viral proteins, evolution of, 527*f*
Viral transduction, 901
Viral vectors, 519, 527–528, 1100
  adeno-associated, targeting of, 505
  engineering, 528–530
  genes, 1232
  transduction, 1231–1232
Viscoelastic material, 1052
Vision enhancement systems, 1163, 1164*f*
  emerging application areas for engineered cells and tissues, 1173–1179

cell matrices, axonal regrowth, 1177–1178
functional outcomes of novel retinal therapies, 1178–1179, 1179*f*
optogenetics, 1174–1177
outer retinal cell transplantation, 1177
photosensitive structures, 1174, 1175*f*
repopulating ischemic/diabetic retina, 1178
vision restoration, 1166–1173
drug delivery, 1172
genetic interventions, 1172–1173
optic nerve protection and regeneration, 1171–1172
optoelectronic and optical means, stimulus through, 1166–1167, 1166*f*
retinal cell transplantation, 1170–1171
visual prostheses, electrical tissue stimulation, 1167–1170, 1168*f*
visual system, architecture, and (dys) function, 1163–1166, 1165*f*
Vision restoration, 1166–1173
drug delivery, 1172
genetic interventions, 1172–1173
optic nerve protection and regeneration, 1171–1172
optoelectronic and optical means, stimulus through, 1166–1167, 1166*f*
retinal cell transplantation, 1170–1171
visual prostheses, electrical tissue stimulation, 1167–1170, 1168*f*
Visual impairment, 1145, 1153
Visually evoked potential (VEP), 1175–1176
Visual-signal processing, 1163
Vitamin A, 1150
Vitamin E, 836*t*, 1095–1096
Vitronectin, 126, 1311, 1314, 1316, 1484
VMCs. *See* Vascular mesenchymal cells
Voltage-gated calcium channels (VGCCs), 1094, 1097
Voluntary activity *versus* evoked potentials, 1038–1040
Voxels, 1395
VSMCs. *See* Vascular smooth muscle cells
VSV. *See* Vesicular stomatitis virus

W

Wake Forest Institute for Regenerative Medicine (WFIRM), 1477
WAP. *See* Whey acidic protein
Warfarin, 638–639
Whey acidic protein (WAP), 99–100
White blood cells (WBCs), 765, 770–779
granulocytes, neutrophils, 778–779
lymphocytes
NK cells, 773–775
NKT cells, 775–776
T cells, 770–773
monocyte-derived dendritic cells, 776–777
monocyte-derived macrophages, 777–778
Whole-body epidermal replacement, 1302
Whole kidney regeneration, 825, 833, 834*t*, 835
decellularization/recellularization strategy for, 834*t*
Whole-lung tissue engineering, 1273, 1275*f*
Whole organ engineering, 8–9, 24, 1280, 1529–1530
Whole tooth-tissue engineering
bioteeth from cell-seeded scaffolds, 1189–1190
cell sources, 1191
root formation, 1190–1191
stem cell-based tissue engineering of teeth, 1189
Wnt3a inhibits osteogenesis, 897
Wnt4, 810–811
Wnt5a, 897
Wnt6 signaling, 84
Wnt9b, 810–811
WNT glycoproteins, 694
Wnt ligands, 712–713
Wnt signaling, 84, 240, 694–695, 712–713, 814–815, 1121–1123, 1140, 1193, 1254–1255, 1277, 1293, 1295, 1297–1298, 1300–1301, 1539
World Organization for Animal Health (OIE), 1369–1370
Wound bed preparation (WBP), 1333, 1341–1342, 1344–1345, 1346*f*
Wound bed score (WBS), 1342, 1346*f*
Wound chamber model, 279
Wound contraction, 1316–1317
Wound healing, 42, 44, 245–246, 246*f*, 364–365, 1049–1050
adhesion and migration, 100–102
apoptosis, 104
in dentin–pulp complex, 1193–1194
differentiation, 103–104
proliferation, 102–103
Wound healing process, 1297–1298, 1333
Wound-induced hair anagen reentry/growth (WIH-A) model, 1300
Wound-induced hair follicle neogenesis (WIHN), 1298
Wound repair, 1309
biology of, 1310–1317
extracellular matrix organization, 1316–1317
granulation tissue, 1312–1316, 1313*f*
inflammation, 1310
reepithelialization, 1310–1312
transition from inflammation to repair, 1310
wound contraction, 1316–1317
chronic wounds, 1317–1318
scarring, 1318–1320
pathological scars, 1318–1319
scarless healing/regeneration, 1319–1320
skin cells, tissue engineered therapy with, 1320–1322
cellular and acellular, 1320
engineered dermal constructs, 1321
engineered epidermal constructs, 1320–1321
engineered skin substitutes, 1321–1322
skin autograft harvesting without scarring, 1322
stem cells, bioactives and biomaterials, 1322–1324
Wounds gain, 1317
Woven 3D fabrics, 162

X

Xenobiotics, 873
Xenogeneic cellular products, 1567
Xenogeneic factors, 852
Xenogeneic material, 1138, 1140
Xenogenic transplantation, 1030
Xenogenic workflows, 1470, 1471*t*
Xenograft, 873, 998–999, 1170
Xenotransplantation, 740, 872–873
X-linked disease, vision enhancement systems, 1173

Y

Yamanaka Cocktail, 1121
Yamanaka factors, 446–447
YH14618, 953

Z

Zika virus, 1542–1543
Zinc-finger nucleases, 520
Zinc-finger transcription factors, 82
Zoledronate, 927
Zwitterionic poly-carboxybetaine conjugates, 522