Principles of Tissue Engineering

Fifth Edition

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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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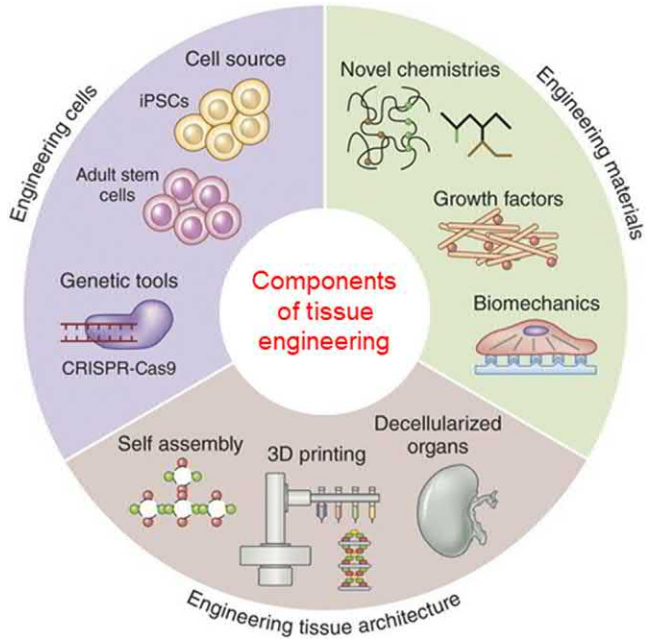


FIGURE 1.1 Schematic representation of different aspects of tissue engineering. Each component (materials, cells, and tissue architectures) can be engineered separately or in combination to achieve the therapeutic goals. *Reprinted with permission from Khademhosseini A., Langer R. A decade of progress 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

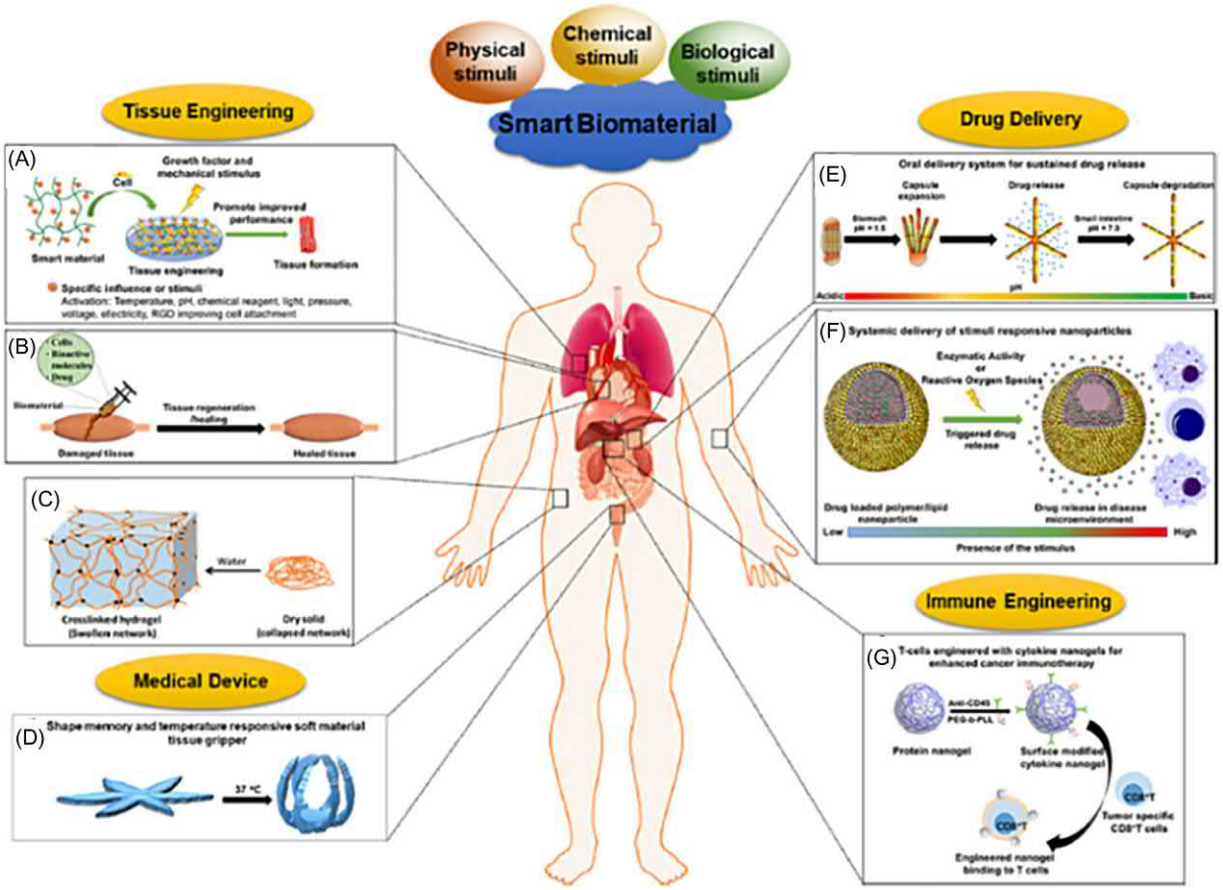


FIGURE 1.2 Different applications of smart biomaterials in the fields of tissue engineering and related fields. (A) Stimuli-responsive material that can promote cell differentiation and tissue growth; (B) injectable biomaterial loaded with cells, drugs, or bioactive molecules can be delivered less-invasively 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

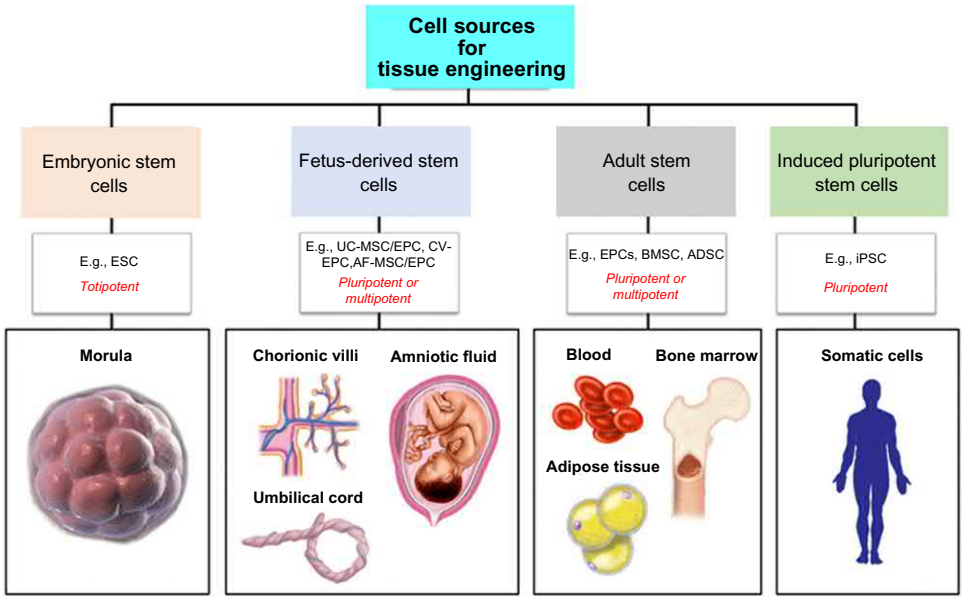


FIGURE 1.3 Different sources of cells for tissue engineering. Fetus-derived and induced pluripotent stem cells are gaining more attention for tissue engineering applications. *Reprinted from Al-Himdani S, Jessop ZM, Al-Sabah A, Combellack E, Ibrahim A, Doak SH, et al. Tissue-engineered solution in plastic 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

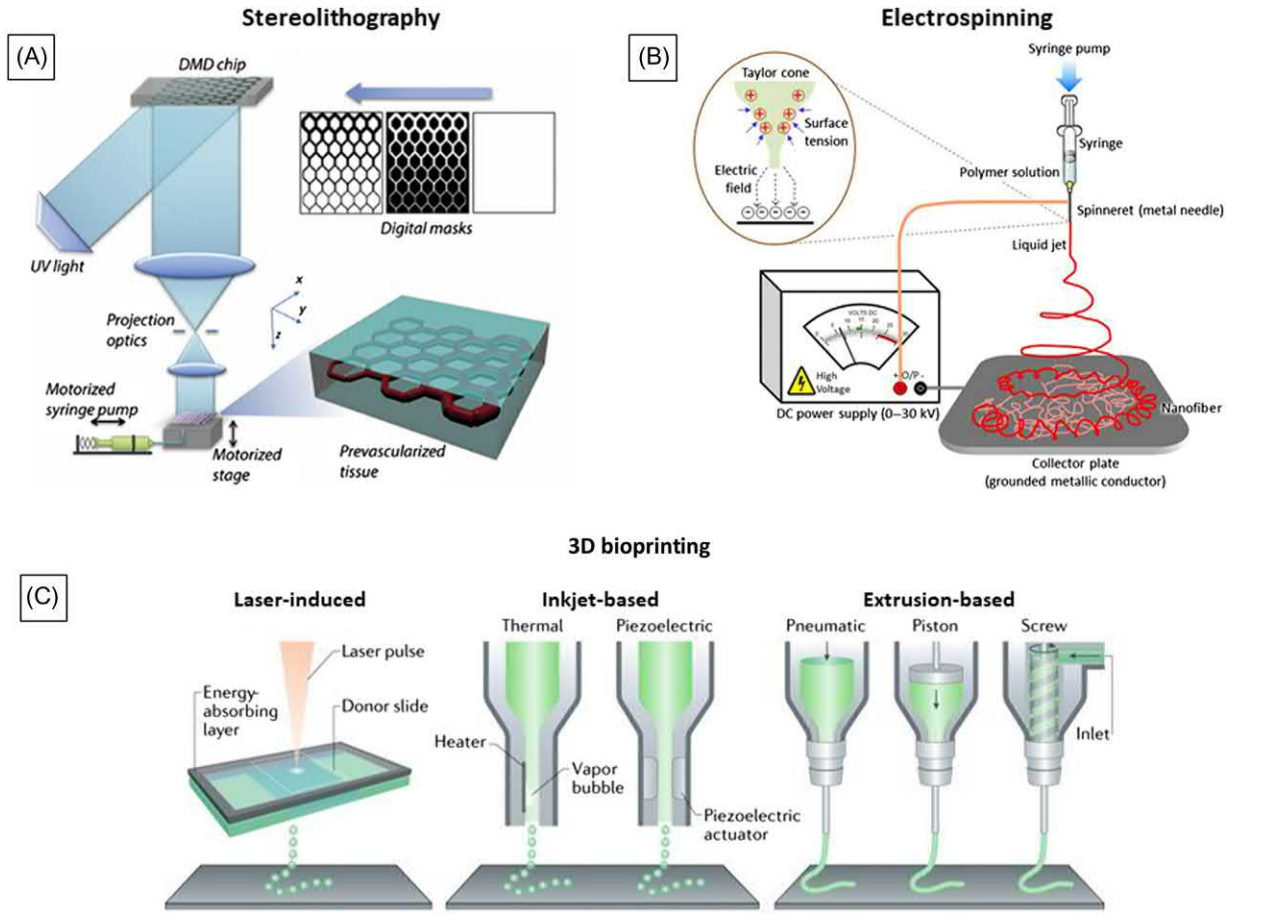


FIGURE 1.4 Different types of biofabrication technologies. (A) Stereolithography, showing an example with bioprinting of a prevascularized tissue; (B) electrospinning; (C) different types of 3D bioprinting, including laser-induced bioprinting, inkjet-based bioprinting, and extrusion-based bioprinting. *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

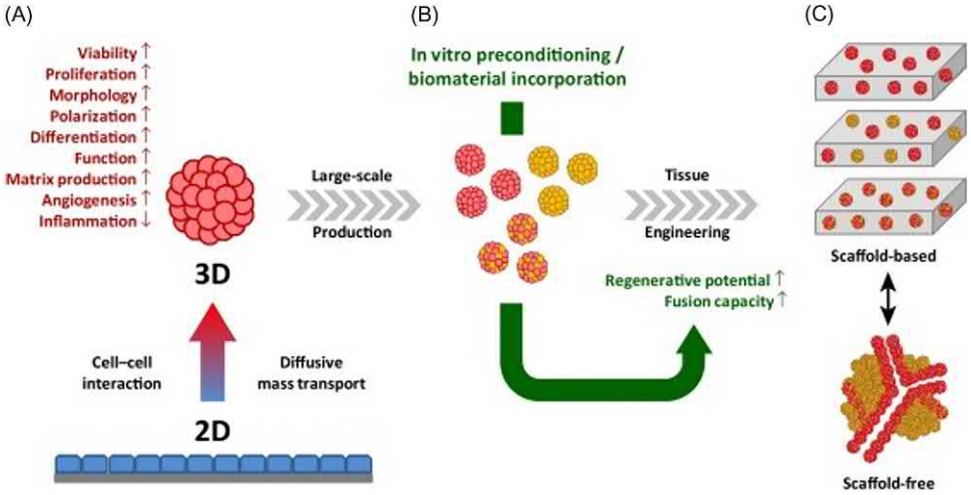


FIGURE 1.5 Use of cellular spheroids, from studying basic biological processes to tissue engineering. (A) Some of the physiological and developmental processes that can be investigated; (B) improving the regenerative potential and fusion capacity of spheroids; (c) the two types of tissue engineering 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

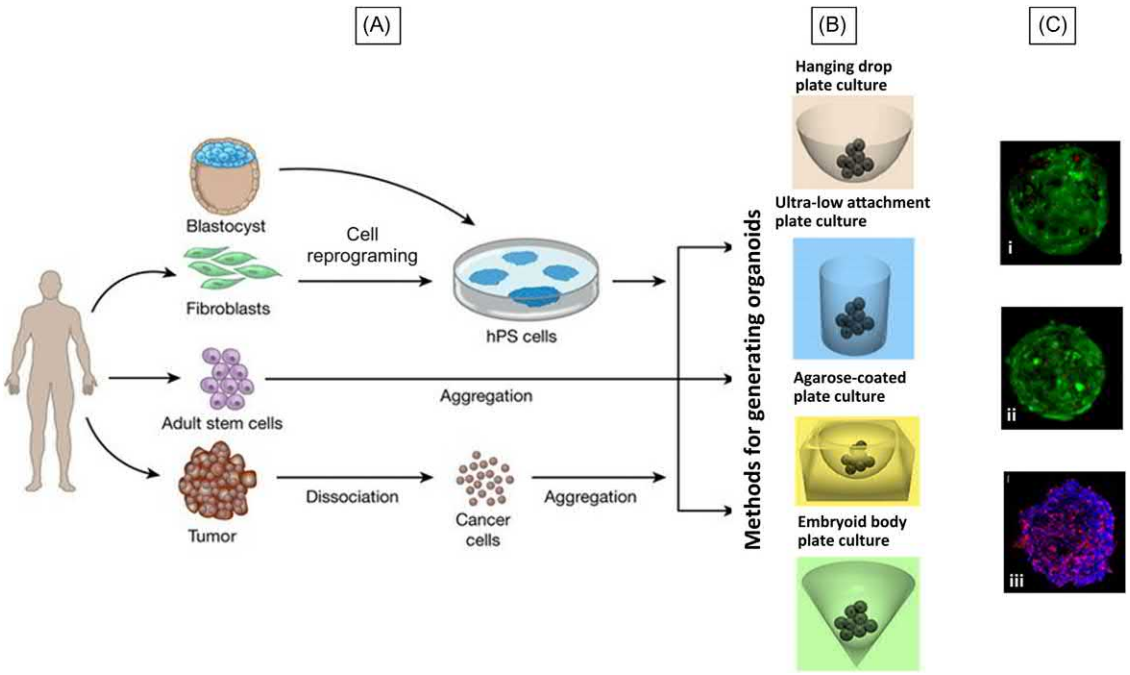


FIGURE 1.6 Different cell sources and methods used to generate organoids. (A) Embryonic stem cells, keratinocyte-derived iPSCs, adult stem cells, or even cells from tumors can be used to make tissue or disease-specific organoids; (B) generating organoids using different platforms; (C) photomicrograph 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].*

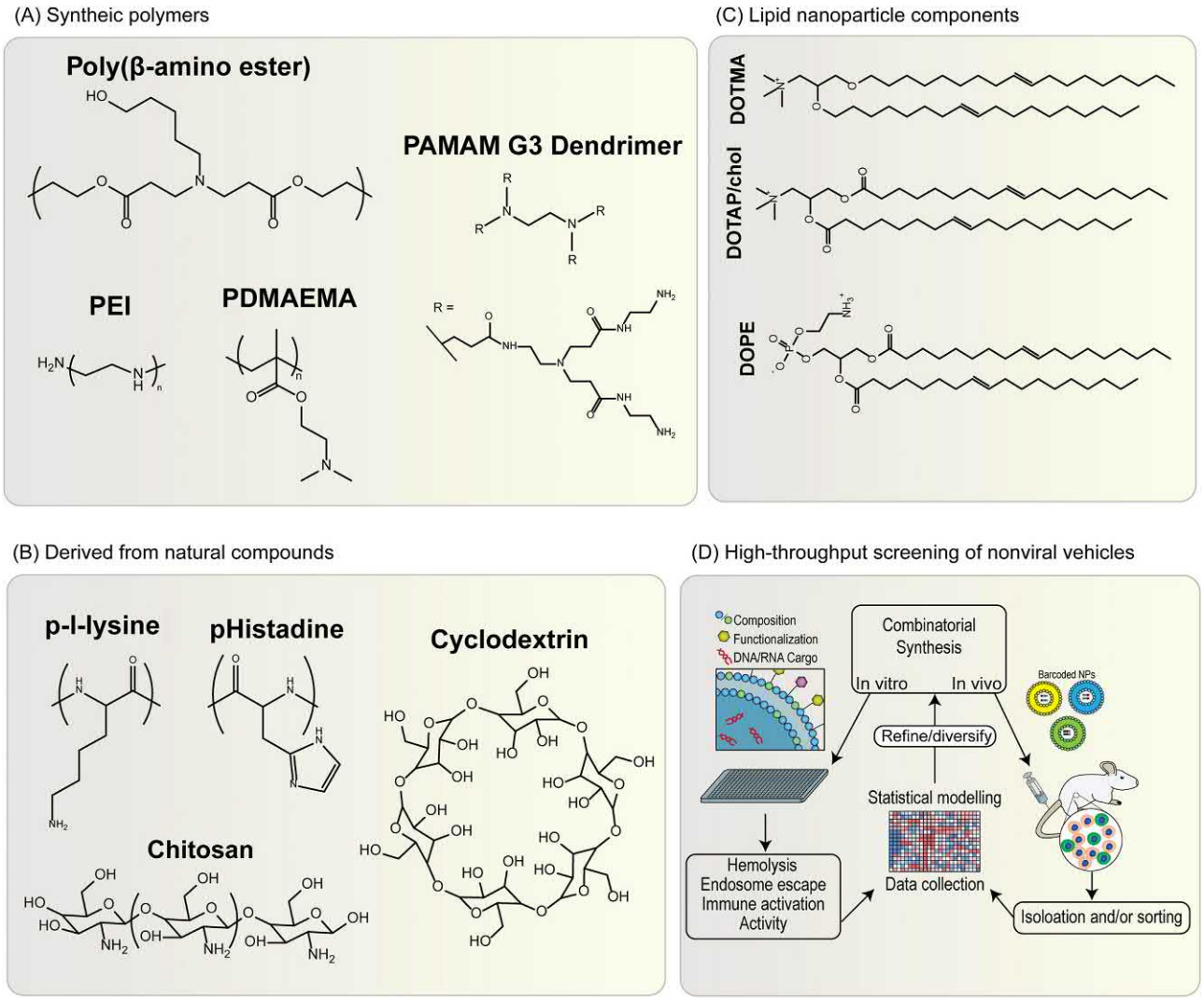


FIGURE 29.7 **Design and optimization of nonviral vehicles for gene delivery.** (A) Several polycationic polymers for nanoparticle production are shown including poly(B-amino esters), PEI, PAMAM dendrimers, and PDMAEMA. (B) Examples of polymers derived from natural components that are used for nucleic 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

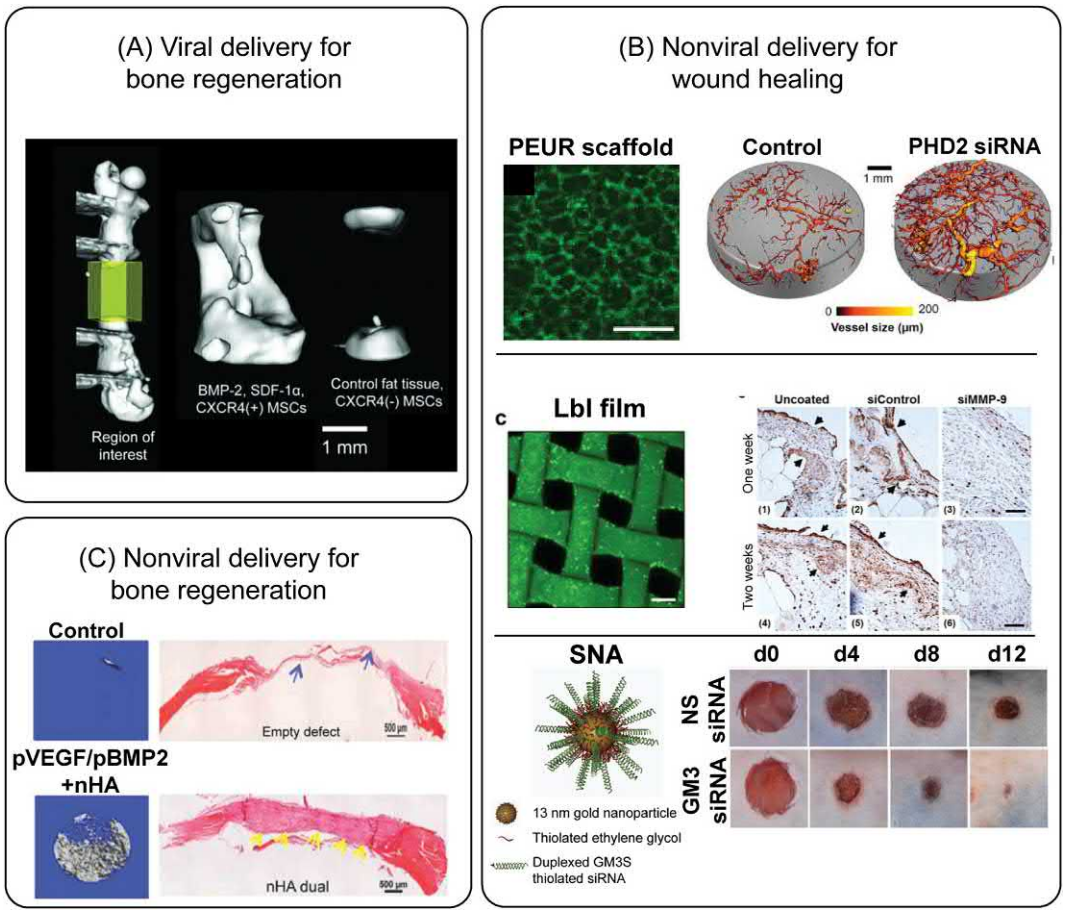


FIGURE 29.8 **Promising preclinical data for tissue engineering includes viral and nonviral delivery.** (A) MSCs transduced to overexpress CXCR4 were implanted and Adenovirus delivering BMP-2 and SDF-1α was delivered showing improvements in bone formation in a critical defect model. (B) Several approaches 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].

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

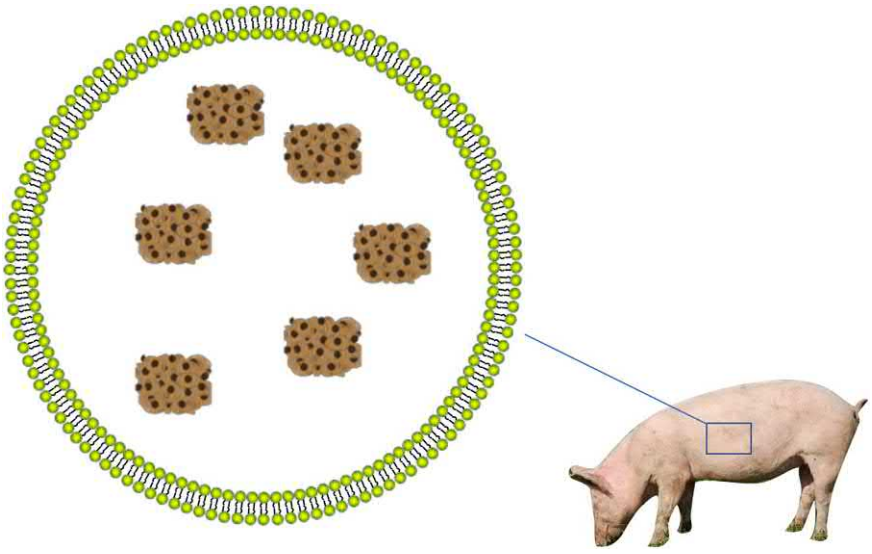


FIGURE 36.1 The concept of cell-encapsulation for immunoprotection was introduced as far back as in 1933. Bisceglie [15] implanted tumor cells after encapsulation in an amniotic sac into the peritoneal cavity of pigs to study the behavior of the cells in the absence of immunosuppression. Bisceglie did not recognize the impact of this approach for treatment of disease.

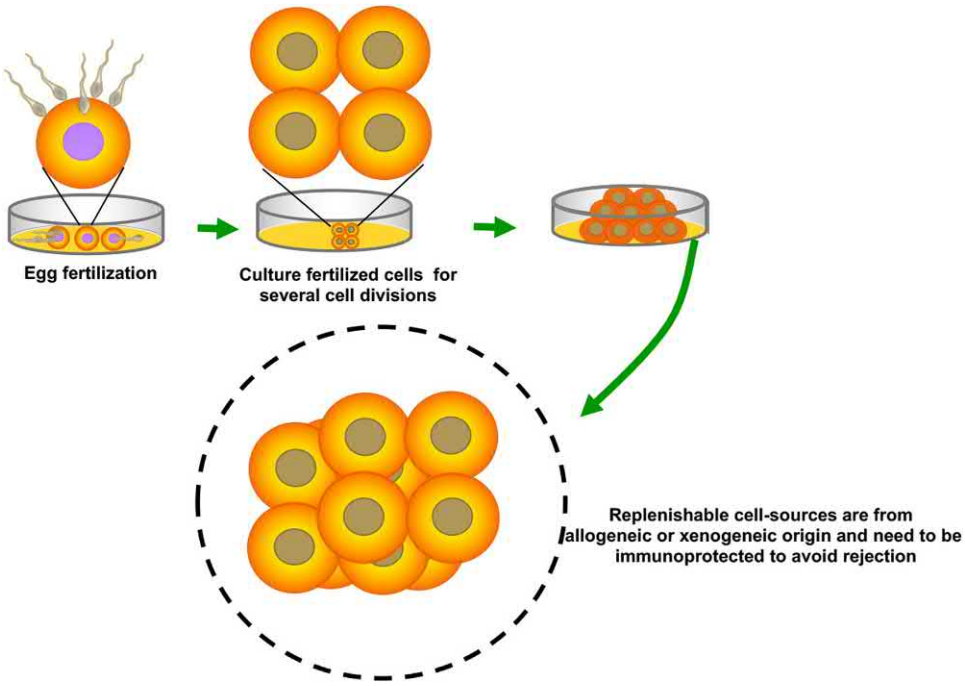


FIGURE 36.2 Replenishable insulin producing cell sources such as cells obtained from embryonic stem cell sources are either from allogeneic or even xenogeneic origin. To prevent graft rejection immunoisolation by encapsulation might be necessary.

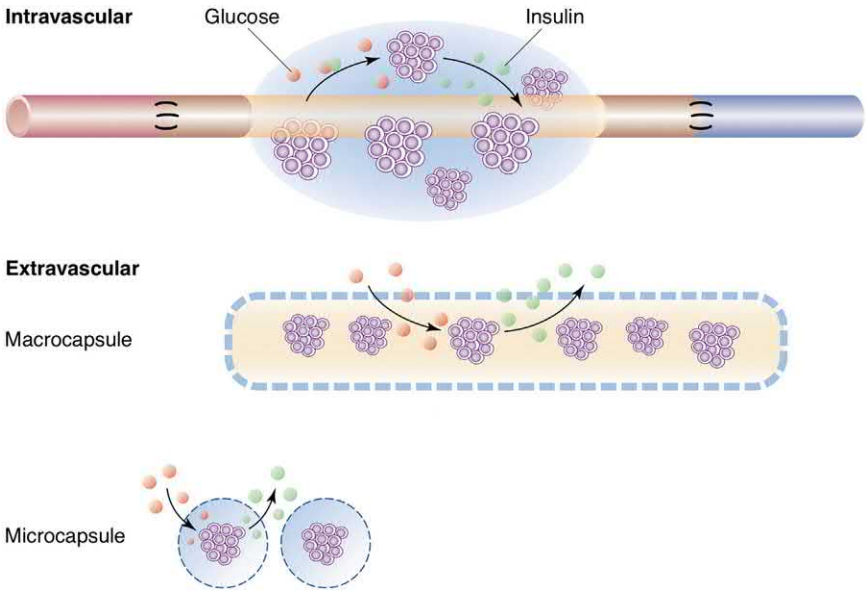


FIGURE 36.3 The bioartificial pancreas exist in three concepts. The intravascular macrocapsules, which allow fast exchange of glucose and insulin due to direct vascular access. The extravascular macrocapsules which can be implanted in the peritoneal cavity or under the skin and the extravascular microcapsules 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

[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

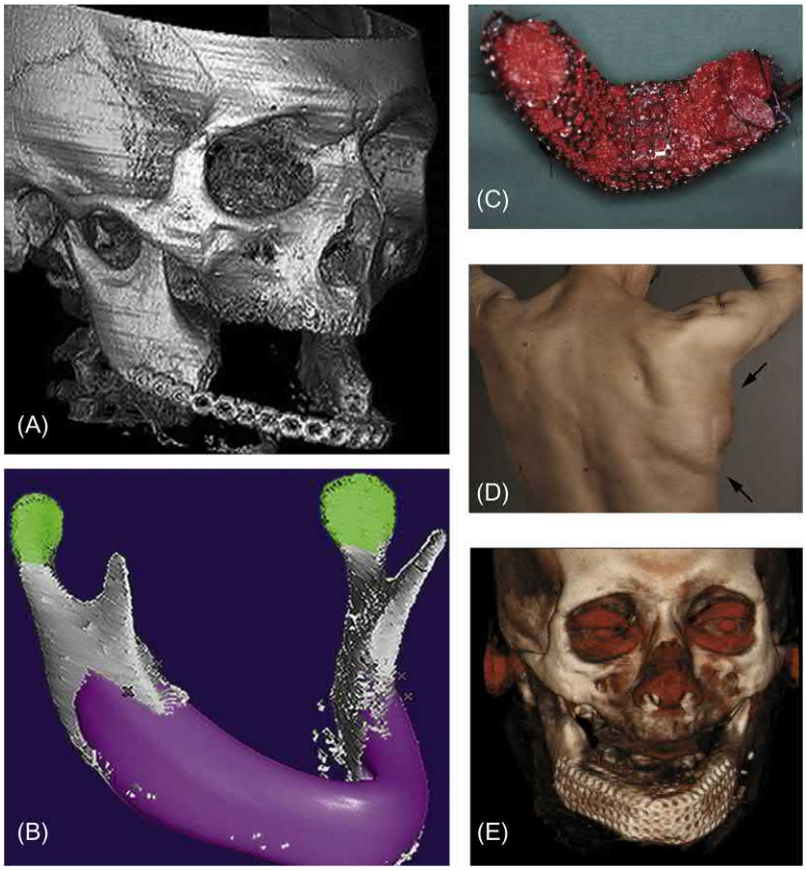


FIGURE 46.1 Image-guided tissue-engineered reconstruction of a massive mandibular defect. (A) The region of interest (jaw) is imaged using 3D CT. (B) The CT data are then fed to CAD software to generate an idealized virtual replacement of the missing parts of the mandible. (C) A titanium mesh is then 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).*

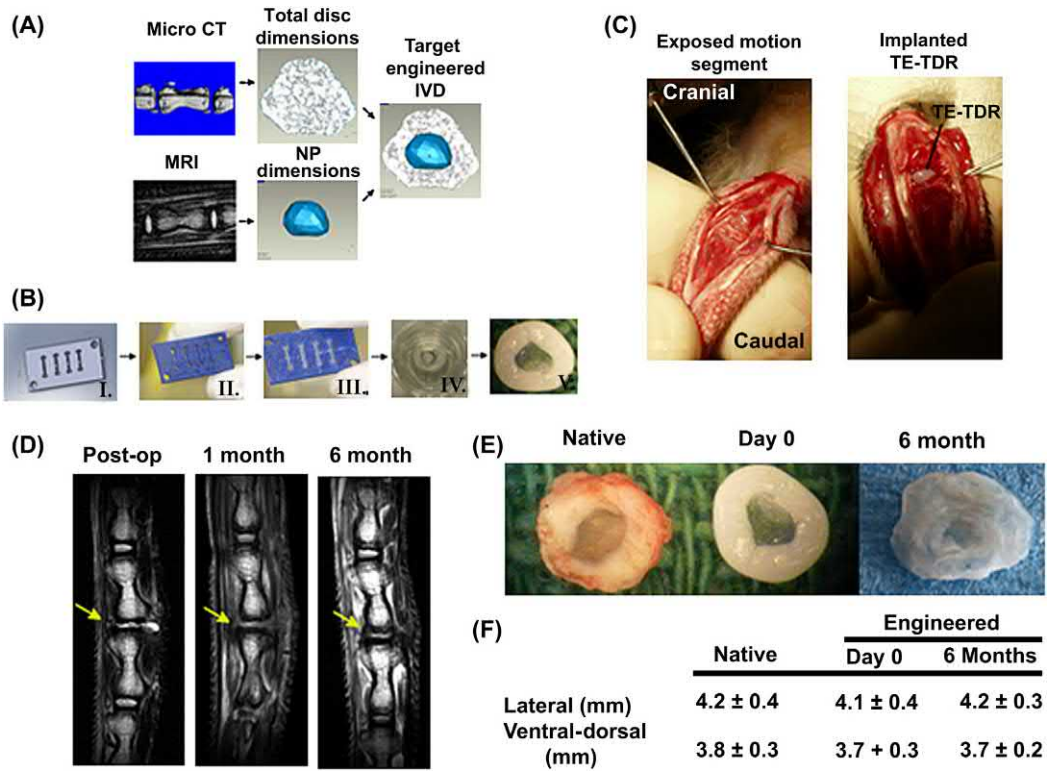


FIGURE 51.7 Anatomical composite TE-IVD, designed from MRI and CT, survives in disk space for 6 months.
(A) CT and MRI design procedure for obtaining TE-IVD dimensions. (B) Fabrication of TE-IVD. (I) NP dimensions used to design injection molds via computer-aided design. (II) Injection mold 3-D printed 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.

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

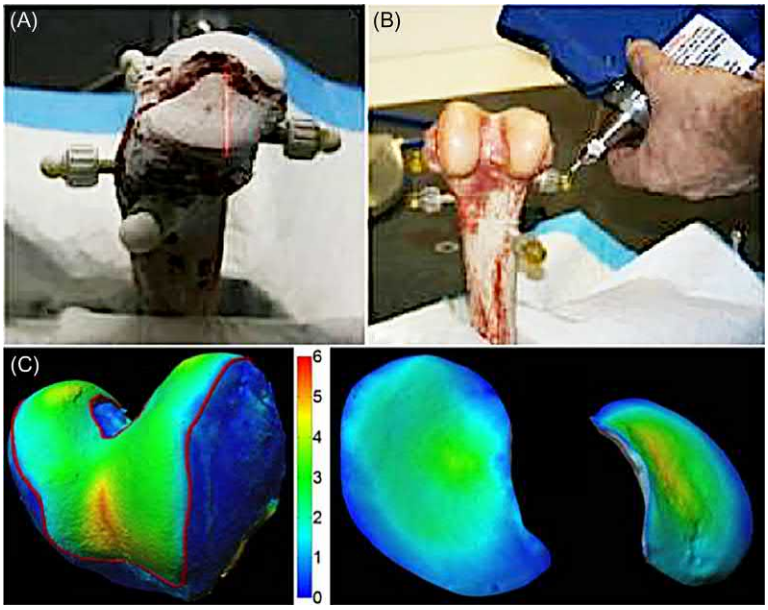


FIGURE 53.1 (A) Laser scanning and (B) physical marker probing of knee specimen [40]. (C) Laser scanner image of cartilage [41].

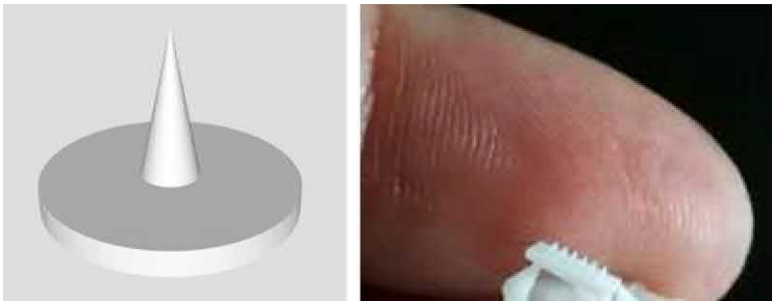


FIGURE 54.8 SLA printed high-resolution microneedles.

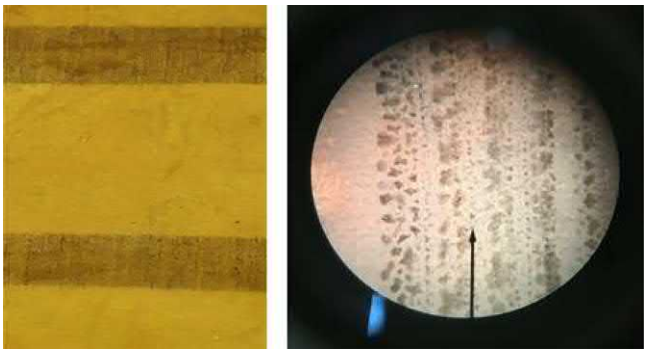


FIGURE 54.9 Inkjet printed graphene oxide–gelatin pattern with high-resolution finer line inside (right:under microscopy).

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

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

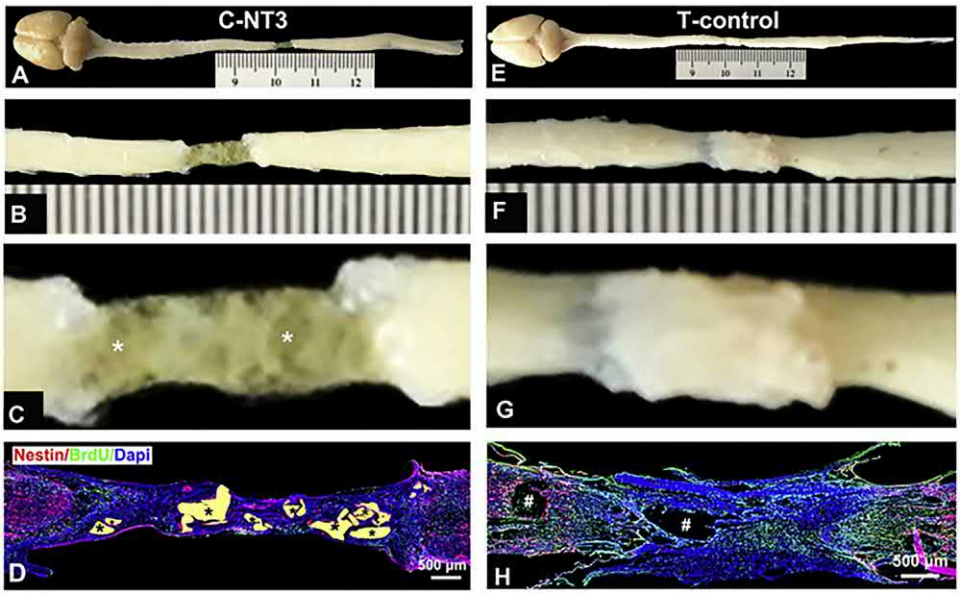


FIGURE 58.13 Validation study of NT-3 releasing chitosan scaffolds. Chitosan tubes filled with NT-3 releasing chitosan carriers were implanted into a 5 mm long segmental transection injury. Gross morphology demonstrated differing appearances of tissue cables after 3 months across the transection gap 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.*

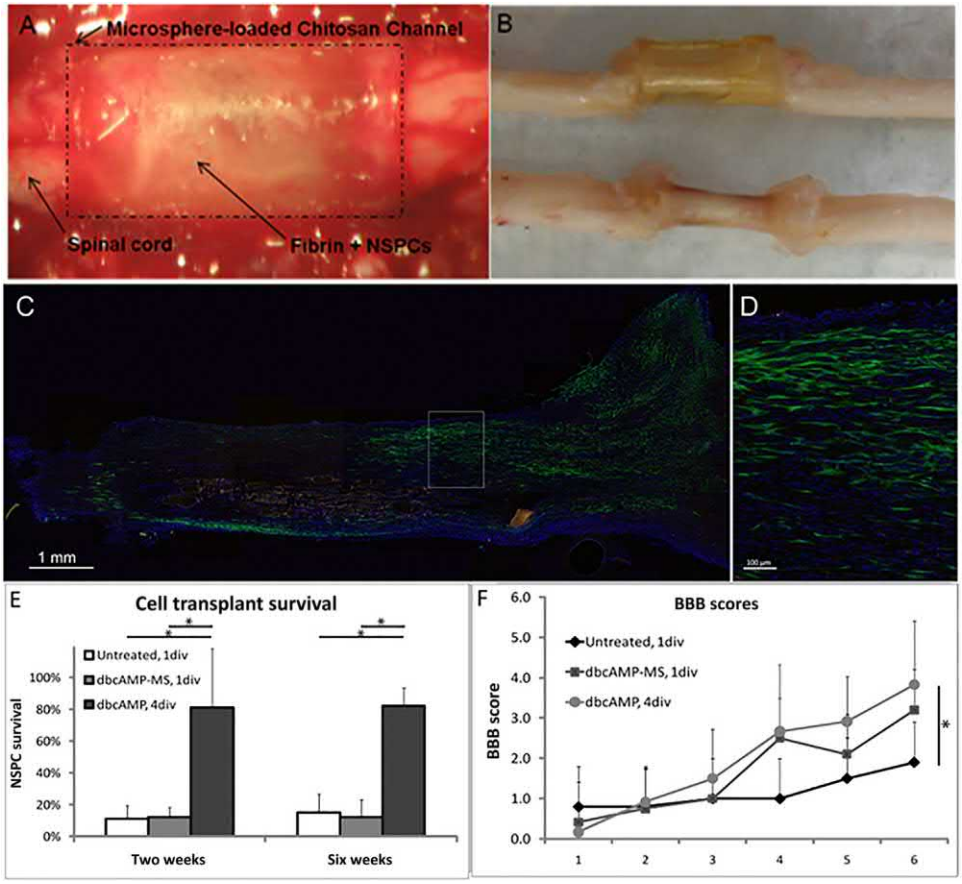


FIGURE 58.14 **Chitosan scaffolds and stem cell transplantation.** A chitosan conduit facilitated spinal cord tissue bridging, NSPC survival, and locomotor behavioral improvement over time. (A) Photographs of the surgical implantation of chitosan channels filled with fibrin and NSPCs also demonstrate (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].

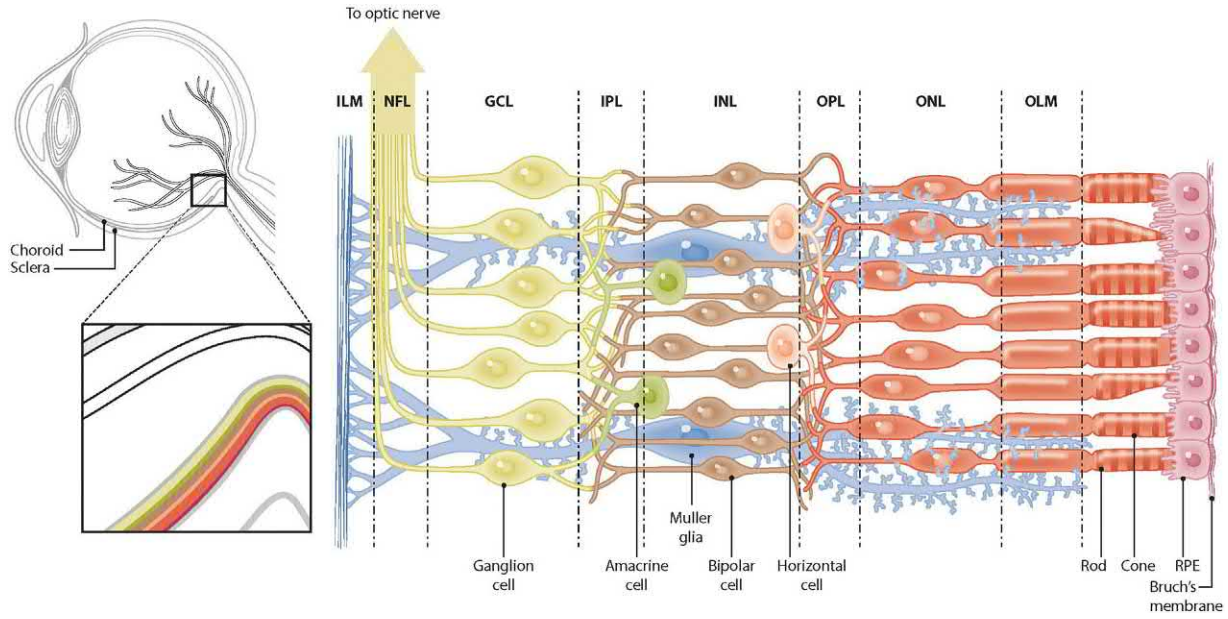


FIGURE 62.1 The eye and the cellular layers of the retina.

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

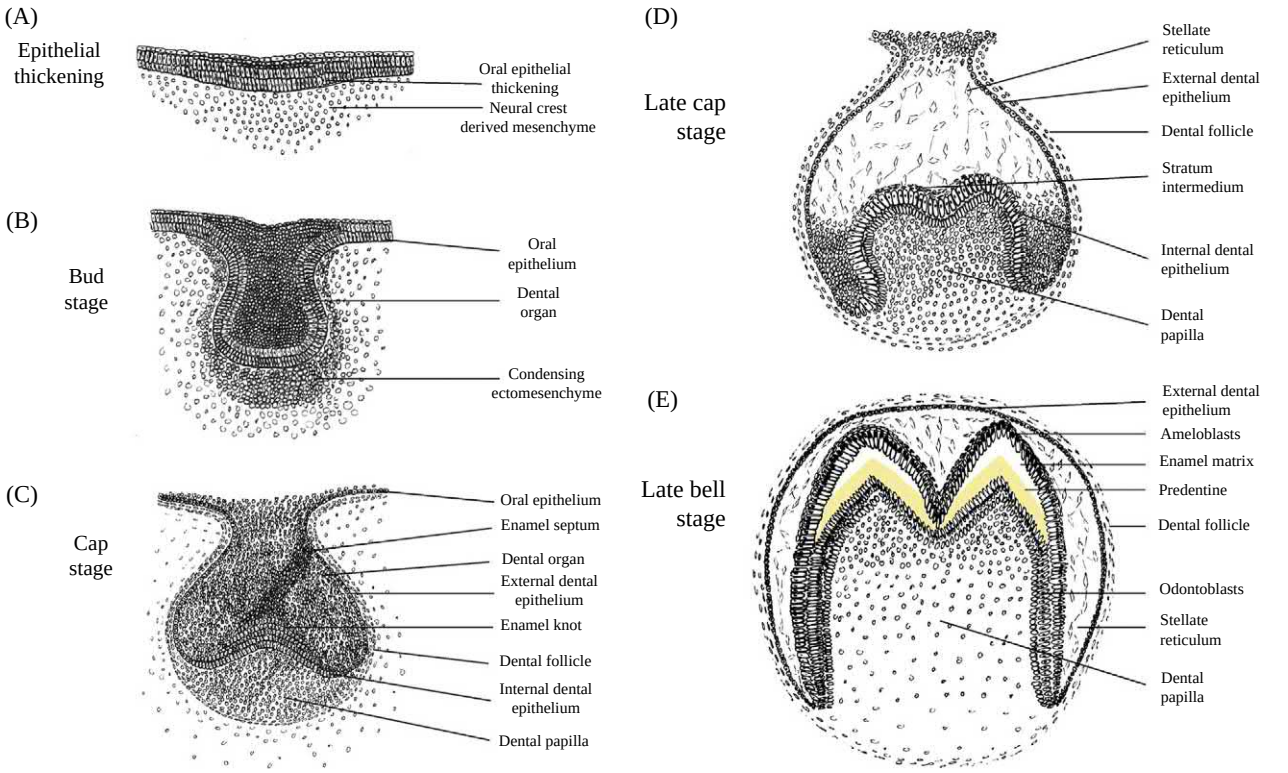


FIGURE 64.1 Drawings of histological sections of mammalian first-molar-tooth development, from the epithelial thickening stage (A) through to the late bell, stage (E).

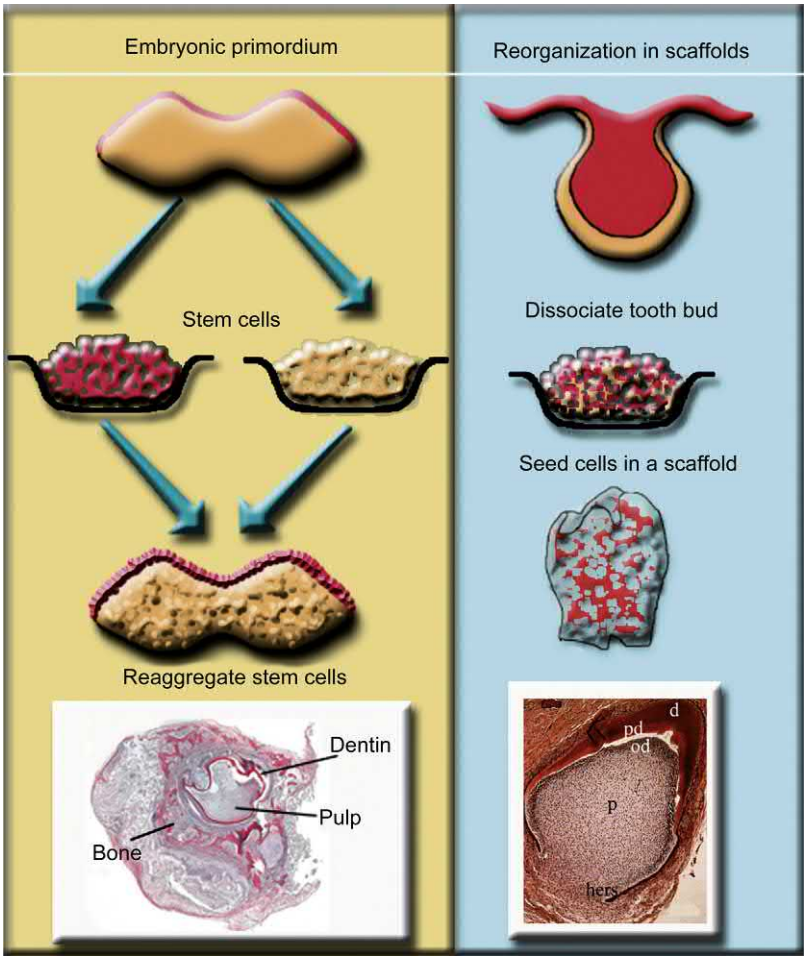
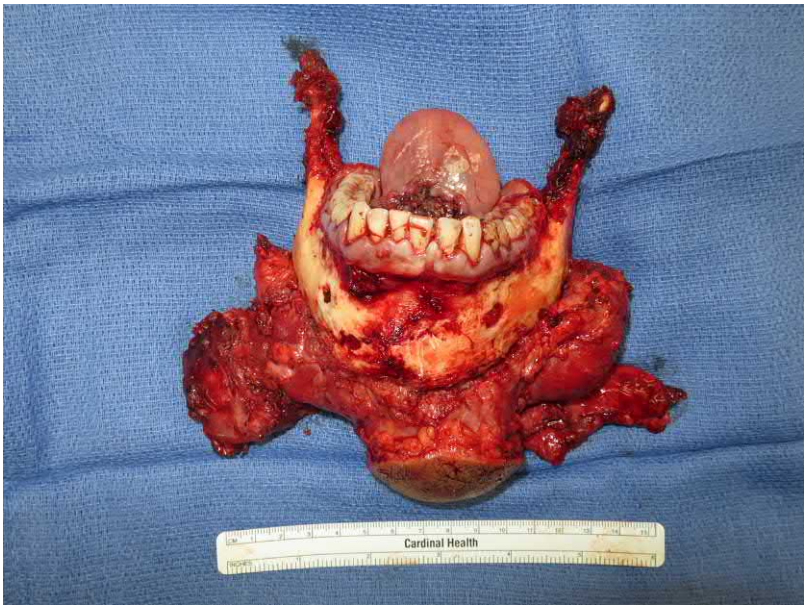
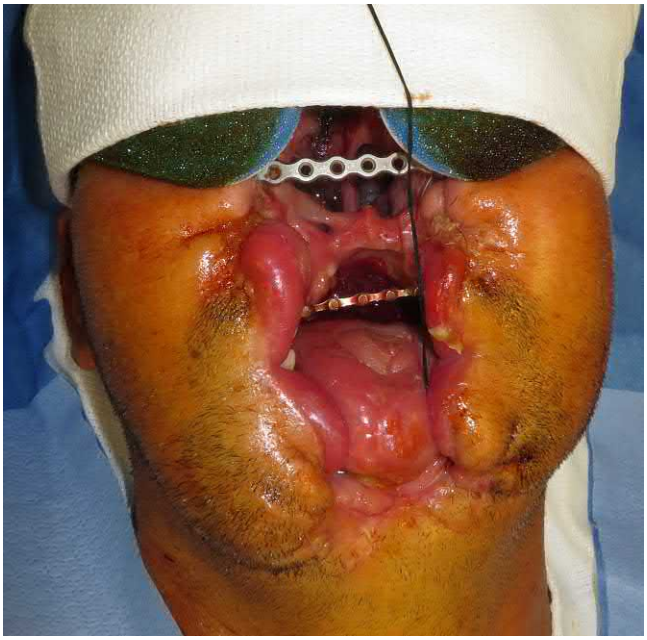


FIGURE 64.2 Diagrammatic representation of two methods currently being explored for producing biological tooth replacement. *Figure kindly drawn by Rachel Sartaj.*



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

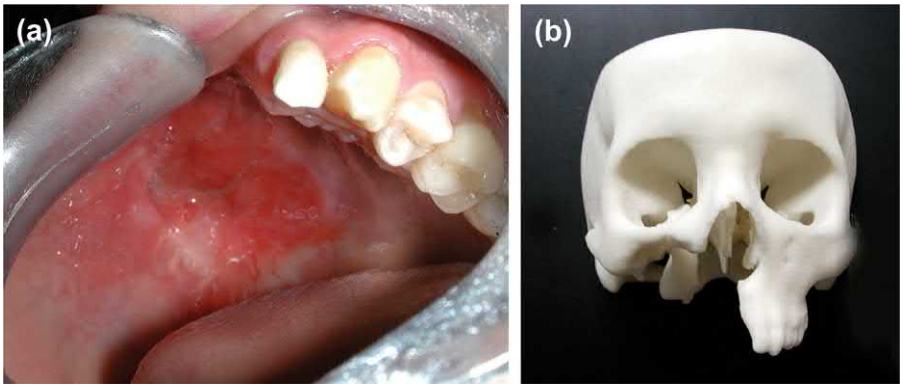
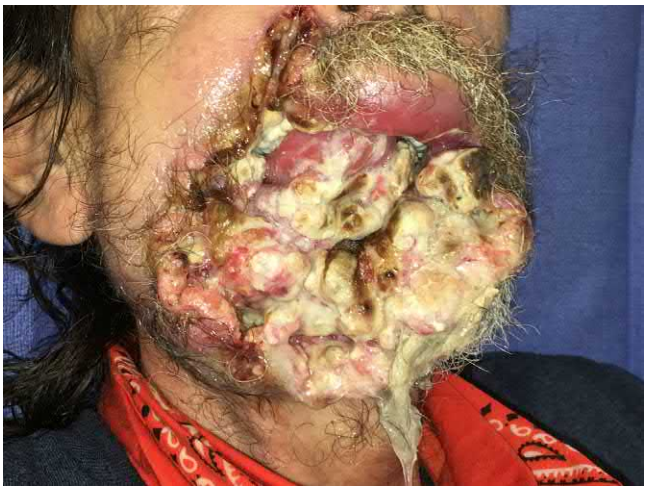


FIGURE 65.5 (A) Patient following a right maxillectomy for removal of a benign odontogenic neoplasm. Defect has filled in with fibrous tissue stimulated by grafting the site with an allogeneic dermal matrix. (B): Stereolithographic model of the same patient demonstrating the extent of the maxillary hard tissue defect.

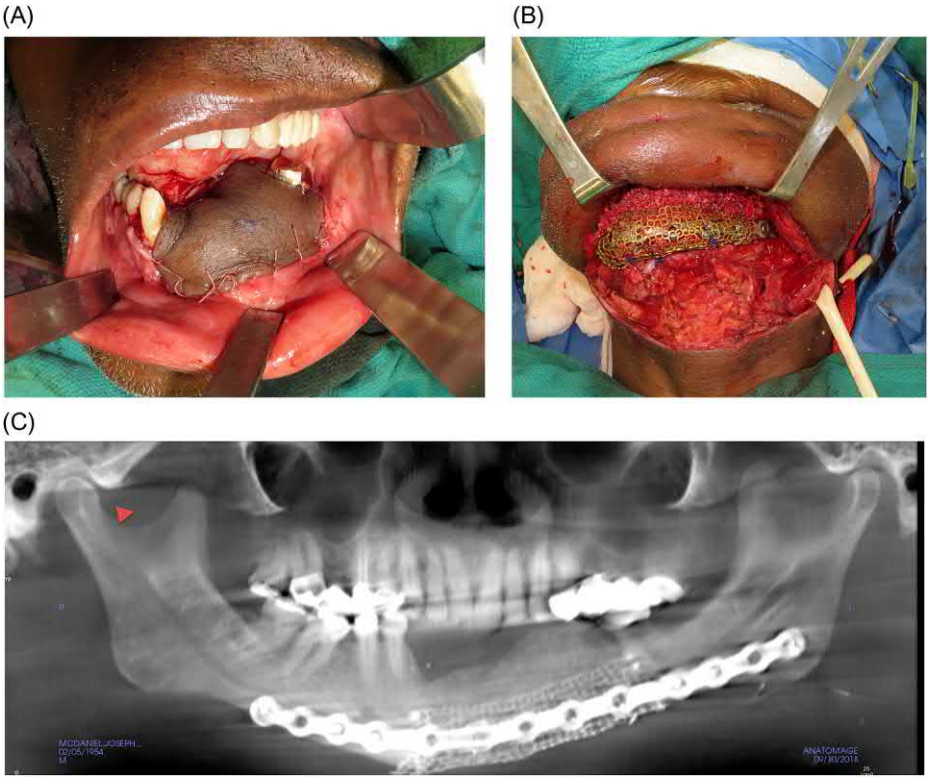
PICTURE 65.6 Placement of avascular bone graft in an infection-free bed via trans-cervical neck incision.



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 65.14 (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

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.

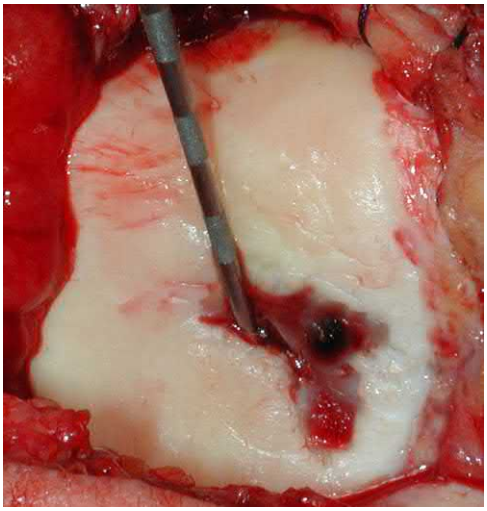


FIGURE 80.1 Cartilage defect of the patella in a 28-year-old man as a result of a direct trauma in the course of a traumatic patellar luxation. Note the partial subchondral involvement indicated by the incomplete blood clot at the base of the defect.

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

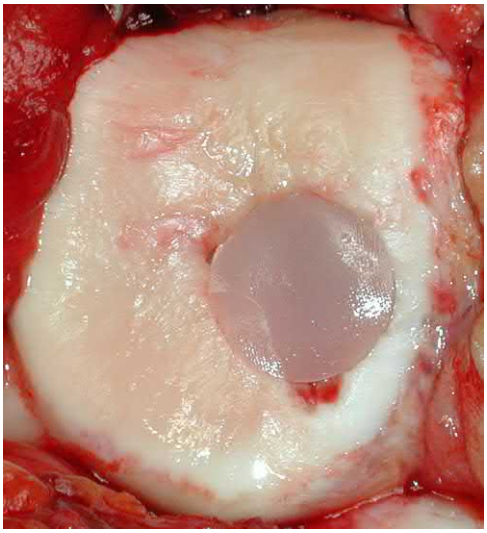


FIGURE 80.2 After meticulous defect preparation, the membrane supporting the chondrocytes in this third-generation ACI product is accurately adapted to the geometry of the defect and implanted. *ACI*, Autologous chondrocyte implantation.

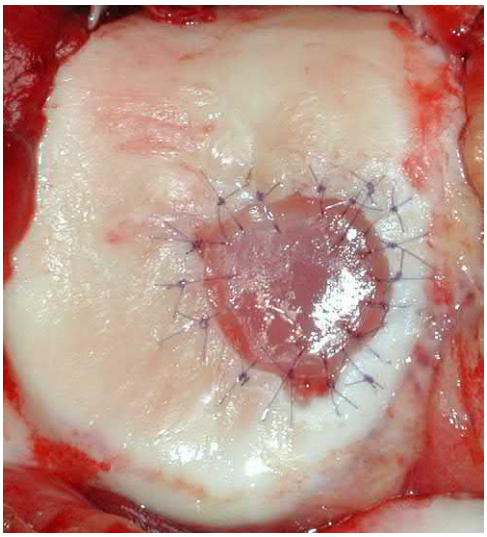


FIGURE 80.3 Fixation of the ACI membrane to the adjacent cartilage with single interrupted sutures (USP 6-0) has been performed. *ACI*, Autologous chondrocyte implantation.

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,"

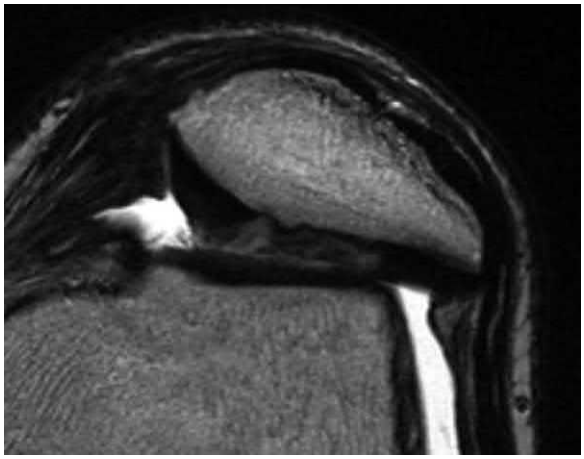


FIGURE 80.4 MRI of the femoropatellar joint of the case after 1 year. Note the complete filling of the defect with a repair tissue, its relatively good integration to the adjacent articular cartilage and the subchondral bone, the irregular subchondral bone plate and the structural differences of the 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