1

Introduction to Tissue Engineering

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1.1 Introduction

Tissue engineering is an interdisciplinary field that utilizes cells, biomaterials, biochemical (e.g., growth factors) and physical (e.g., mechanical loading) signals, as well as their combinations to generate tissue-like structures [1]. The goal of tissue engineering is to provide biological substitutes that can maintain, restore, or improve the function of damaged tissues [2]. Although the first tissue-engineered skin products were introduced in the late 1970s and early 1980s giving rise to modern tissue engineering, the term "tissue engineering" was coined only in 1987 [3–6].

In fact, the use of prosthesis (e.g., gold for tooth replacement and wood for limbs and toes) was employed as early as ancient Egyptians. However, these treatments were all based on nonliving materials, which provided some structure and function but were very far from the original tissue. Medical development led, in the middle of the twentieth century, to the possibility of replacing an entire organ with an organ from a donor, known today as *organ transplantation* [7]. Although this is widely practiced today and is known to be the ultimate solution for organ failure, the need for organs always surpasses the number of available donated organs [8]. The limited donor availability and rejection of the grafts by the immune system drove the concept of *in vitro* grown tissues. The success in tissue engineering of skin grafts boosted the interest in applying similar concepts to other tissues and organs [9]. However, the relatively simple structure, the limited vascular demands of skin, and the ease of growing keratinocytes *in vitro* are not common to most tissues. The dream of regenerating tissues *in vitro* faced major hurdles associated with the engineering of complex, three-dimensional (3D), vascularized multicellular tissues.

In this chapter, we provide a brief introduction to tissue engineering. The clinical needs for tissue engineering, the history, the fundamentals, and the applications of tissue engineering are discussed in brief. The recent advancements in the

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field, as well as some of the major challenges and the future of tissue engineering, are also briefly discussed.

1.2 Clinical Need for Tissue Engineering and Regenerative Medicine

The clinical need for tissue engineering and regenerative medicine is the result of our urge to treat defective tissues. Regardless of how such defects occurred (congenital or acquired), traditional medical tools are not yet capable of completely or efficiently fixing them. In fact, traditional medicine has severe limitations in delivering solutions for numerous health problems. Injuries and diseases are traditionally treated using pharmaceuticals, whereas prosthetic devices and organ transplantation are used in more severe conditions. While pharmaceuticals may be useful for the treatment of numerous conditions, they cannot cure a number of deadly diseases (e.g., several forms of cancers, strokes, diabetes, etc.) or diseases at their advanced stages (e.g., Alzheimer's, Parkinson's, osteoarthritis, etc.). On the other hand, prosthetic devices are not capable of restoring normal function, and the number of organ donors is always way less than required. Tissue engineering can be used to treat diseases that cannot be cured with regular pharmaceuticals and to provide natural, living, functional organs to overcome the need for donors and prosthetics.

The main goal of tissue engineering is the development of functional substitutes for damaged tissues [2]. It is estimated that the majority of tissue engineering products are used for the treatment of injuries and congenital defects, while tissue engineering products used for the treatment of diseases are less common. The worldwide tissue engineering and cell therapy market has been estimated in 2014 at about $15 billion and is expected to grow up to $32 billion by 2018. The dominant market is in the orthopedic, musculoskeletal, and spine areas followed by the skin, nervous tissues, and other organs [10]. Skin was the first tissue to be engineered; this is because of the relatively simple structure of the tissue (can be prepared using two-dimensional (2D) culture and has easy access to culturing medium). Skin is also an important tissue engineering target because of the high demand especially resulting from war burns. Skin damage can cause disfigurement and disability, which may lead to further serious infections and psychological damage to patients. All these factors made skin one of the first clinical tissue engineering targets. Tissue engineering and regenerative medicine solutions can also be applied for any tissue, although the levels of complexity would differ between targets. Examples include the heart, kidneys, cornea, nervous tissues, liver, intestines, pancreas, lungs, bone, muscle, and so on. The ultimate goal is that tissue engineering and regenerative medicine would one day be able to overcome the need for organ transplantation. The medical need for tissue engineering and regenerative medicine can be emphasized in the donor waiting list, which is always increasing at a higher pace than the number of organ donors. The ability to engineer such organs or help them regenerate would represent a great leap in the history of the health care field.

1.3 History of Tissue Engineering and Regenerative Medicine

Generating new tissues and restoring body parts or organs are ideas that were embedded in humans' imaginary world from the dawn of history. The revolution of the human race enabled these imaginary notions to become well-practiced findings all over the years. In the case of the ancient Egyptians, restoring body parts was reasoned by the importance of reuniting and reassembling the body to enable revitalization in the Afterlife, as inscribed in spells known as the "Pyramid Texts" (2375 BC) [11]. It is believed that the first dental prosthesis was constructed from gold in Egypt around 2500 BC [12]. Nerlich and colleagues account for an ancient Egyptian false big toe believed to be the oldest limb prosthesis (950–710 BC), Figure 1.1a [13]. Interestingly, this prosthesis was recently found to improve function and walking, which indicates the possibility that the purpose of these designs was not only for the Afterlife [14].

The use of nonliving materials enabled the restoration of the structure, shape, and function to some extent. However, living tissues would be needed to achieve a full recovery. History notes the miraculous leg transplantation by Saints Cosmas and Damien (about 287, Figure 1.1b). In the sixteenth century, Gaspaire Tagliacozzi Bologna, Italy, was the first to write a book on plastic surgery where he first described the nose reconstruction from the forearm flap. Tagliacozzi made a great revolution at that time when alterations in body appearance were religiously prohibited [15].

The progress in anesthesia and infection prevention in the nineteenth century helped in the rapid development of surgical procedures. This development allowed the first applications of living tissues and organs to recover malfunction [16]. Skin grafts were the first tissue-based therapies, and the introduction of techniques to preserve cells and tissues enabled allograft skin banking [17–19]. Shortly thereafter, the first successful complete organ transplantation of a kidney

Figure 1.1 Some random images showing the development of regenerative medicine throughout different eras in history. (Nerlich 2000 [13]. Reproduced with permission of Elsevier.) (a) 2500 BC: false big toe developed in ancient Egypt. (b) 278 AD: Saints Cosmas and Damian performing a leg transplant from a deceased donor onto a patient with an amputated leg. (Zimbler 2001 [15]. Wikipedia, public domain, https://commons.wikimedia.org/wiki/File:Fra_Angelico_064.jpg.) (c) In 2013, Chinese doctors saved a man's severed hand by grafting it to his ankle before later reattaching it to the patient's arm. (Gordon 2006 [21]. Reproduced with permission of John Wiley and Sons.)

between identical twins could be achieved [20]. Limited donor availability and rejection of the grafts by the immune system drove the concept of *in vitro* grown tissues, giving rise to the field of "tissue engineering."

The success of engineering skin grafts boosted interest in applying similar concepts to other tissues and organs. However, the relatively simple structure, limited vascular demands of skin, and the ease of growing keratinocytes *in vitro* are not common to most tissues. Tissue engineering first raised immense public awareness and media interest in 1997 when the BBC documented the potential of engineering an ear (Figure 1.3) [22]. The so-called Vacanti mouse represented the promise that tissue engineering holds for tissue recovery becoming known by millions around the globe. Despite the innovative and exciting nature of the Vacanti experiment, it represented only the beginning of the tissue engineering journey and the organ engineering "proof of concept." The engineered tissue in the Vacanti experiment had many limitations that make it difficult if not impossible for the system to be translated to a clinical scenario without major alterations. The engineered ear, which was intended for a three-year-old boy, was prepared using a polyglycolic acid (PLA) scaffold seeded with bovine chondrocytes and implanted in an immunodeficient mouse for culture. If such a tissue is implanted in a human, it would result in a strong immune response not only due to the mouse where it had been grown but also due to the cultured bovine cells. The ideal replacement for the mouse would be a human, most ideally the ear receiver, to totally reduce immune rejection. On the other hand, the replacement for the bovine cells would be autologous chondrocytes, which are very limited in supplies. Alternatively, other cell sources may be used, all presenting their advantages and disadvantages, which will be discussed in more details in the following sections. A second limitation is the skin coverage of the engineered tissue, which would either be missing if only the scaffold/cell structure is extracted or would have immune and structural limitations if it is removed together with the mouse skin. Fixing the skin coverage limitation might be possible using skin grafting, but it would highly increase the complexity of the system. A third limitation is concerned with the control over the growth of the ear during the mouse culture period and after transplantation. Further limitations concerning the mechanical and chemical properties of the engineered tissue resulting from the scaffold and culture conditions are all issues that face tissue engineering even today. The dream of regenerating tissues *in vitro* faced and is still facing major hurdles associated with the engineering of complex three-dimensional (3D) vascularized multicellular tissues.

1.4 Fundamentals of Tissue Engineering and Regenerative Medicine

1.4.1 Tissue Engineering versus Regenerative Medicine

Tissue engineering and regenerative medicine are often used interchangeably. However, tissue engineering typically involves the construction of a tissue *in vitro*, while regenerative medicine refers to tools for helping the body

regrow a damaged tissue *in vivo* in the patient. The need for cell sources in tissue engineering was a major limiting factor in the advancement of the field. This shortage of cell sources ignited the use of renewable cells such as stem cells and progenitors, leading to the term "regenerative medicine." Regenerative medicine is mostly based on understanding morphogenesis and natural, inherent self-repair mechanisms, and, as such, regenerative medicine typically involves the use of stem cells and progenitors. Tissue engineering and regenerative medicine, often abbreviated as "TERM," are today complementary. There is an increased interest in the use of various stem cell sources and a need to reduce culture times for engineered tissues, which consequently results in a shorter waiting period and lower prices. This will eventually result in strengthening the bonds between tissue engineering and regenerative medicine, which are likely to become inseparable.

1.4.2 The Triad of Tissue Engineering

Tissue engineering applications typically involve the combination of three pillars: cells, signals, and scaffolds, which represent the "triad of tissue engineering" (Figure 1.2). Although many of the claimed tissue engineering applications might lack one of these pillars, their combination appears to be essential for the success of tissue engineering applications. Current advances in tissue engineering involve developments in all elements of the triad. In this section, major advances in cell

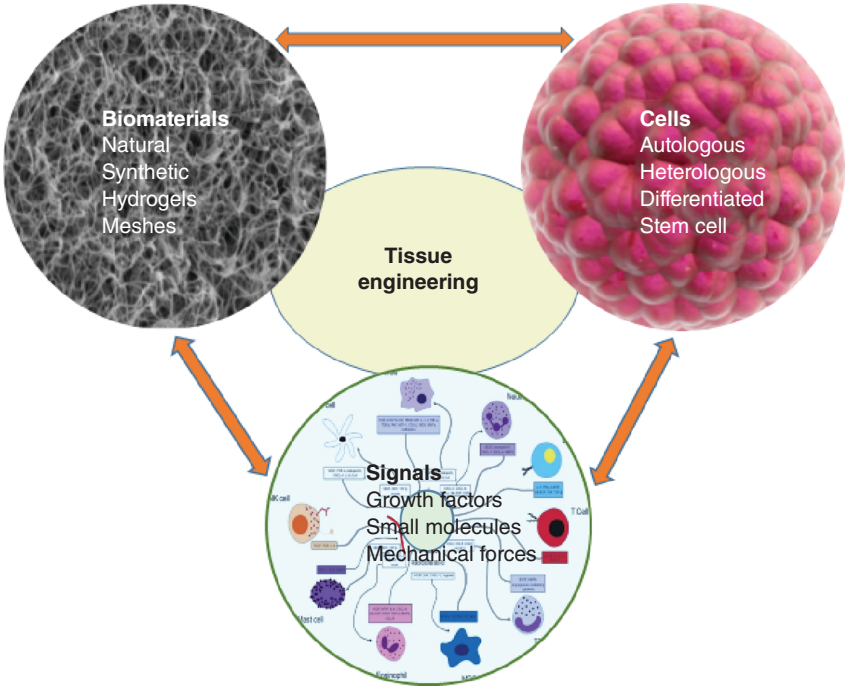

Figure 1.2 The triad of tissue engineering. The combination of cells, scaffolds, and signals is used to engineer functional tissues.

sourcing, scaffold production, signaling in tissue-engineered structures, and their combinations to create functional tissues and organs will be presented.

1.4.3 Approaches in Tissue Engineering

Incorporating the three elements of tissue engineering needs a good scaffolding technique. Over the years, different approaches have been developed, resulting in scaffolds that can support the cells and encourage tissue growth after implantation (Figure 1.3).

The most common approach is the use of a pre-made porous scaffold. Using raw materials – which can be either natural or synthetic – a porous scaffold is created through one of the different fabrication technologies currently available. The diverse possibilities of biomaterials to use and the ability to design the scaffold in a way to control its physicochemical properties make this method especially advantageous. Examples of manufacturing techniques that are used include porogens or fiber-based techniques as well as new solid free-form technologies. Once the supporting scaffold is ready, cells can be seeded inside or on top of the scaffold. A disadvantage of this method is that the post-fabrication cell seeding is both time consuming and not very efficient [23].

Instead of seeding the cells in the scaffold after it is fabricated, another strategy is to encapsulate the cells during scaffold formation. While the number of biomaterials that can be used to create this type of scaffolds is more limited, an advantage is the possibility of delivering the cells in a liquid precursor *in vivo*. Hydrogels (natural or synthetic) are usually used as a scaffold material for encapsulation given their biocompatibility and mild gelation conditions [24]. However, given the poor mechanical properties of hydrogels used in this application, this scaffolding approach is rarely used for tissues having load-bearing functions [23].

Another method that can be used for scaffolding is the decellularization of the extracellular matrix (ECM) from either allogeneic or xenogenic tissues. The ECM is a natural scaffold that allows cell attachment, proliferation, and differentiation. When seeded with the proper cells, it can produce an autologuous construct without the need for extracting tissues from the patient him/herself [25]. The advantages of this method are that it is biocompatible and presents the closest natural mechanical and biological properties needed in the body. The main disadvantage of these systems is the limited supply of autologous tissues and immune responses to non-autologous tissues. Additionally, some minor problems still exist such as inhomogeneous distribution of the seeded cells and the difficulty of removing all immune-provoking material [23]. This technique has proven useful in skin, bladder, and heart valve repair. It has also produced many commercialized decellularized scaffolds with the U.S. Food and Drug Administration (FDA) approval to be used in humans [25].

A final approach is the use of cell sheets prepared using temperature-responsive culture dishes, in a technique known as *cell sheet engineering*. This method avoids the problems caused by transplanting engineered tissues based on fabricated scaffolds; in fact, after the scaffold degrades in the body, it is often replaced by autologous ECM, which can cause fibrosis. In addition, some properties of scaffolds might be undesirable for specific applications. With the development

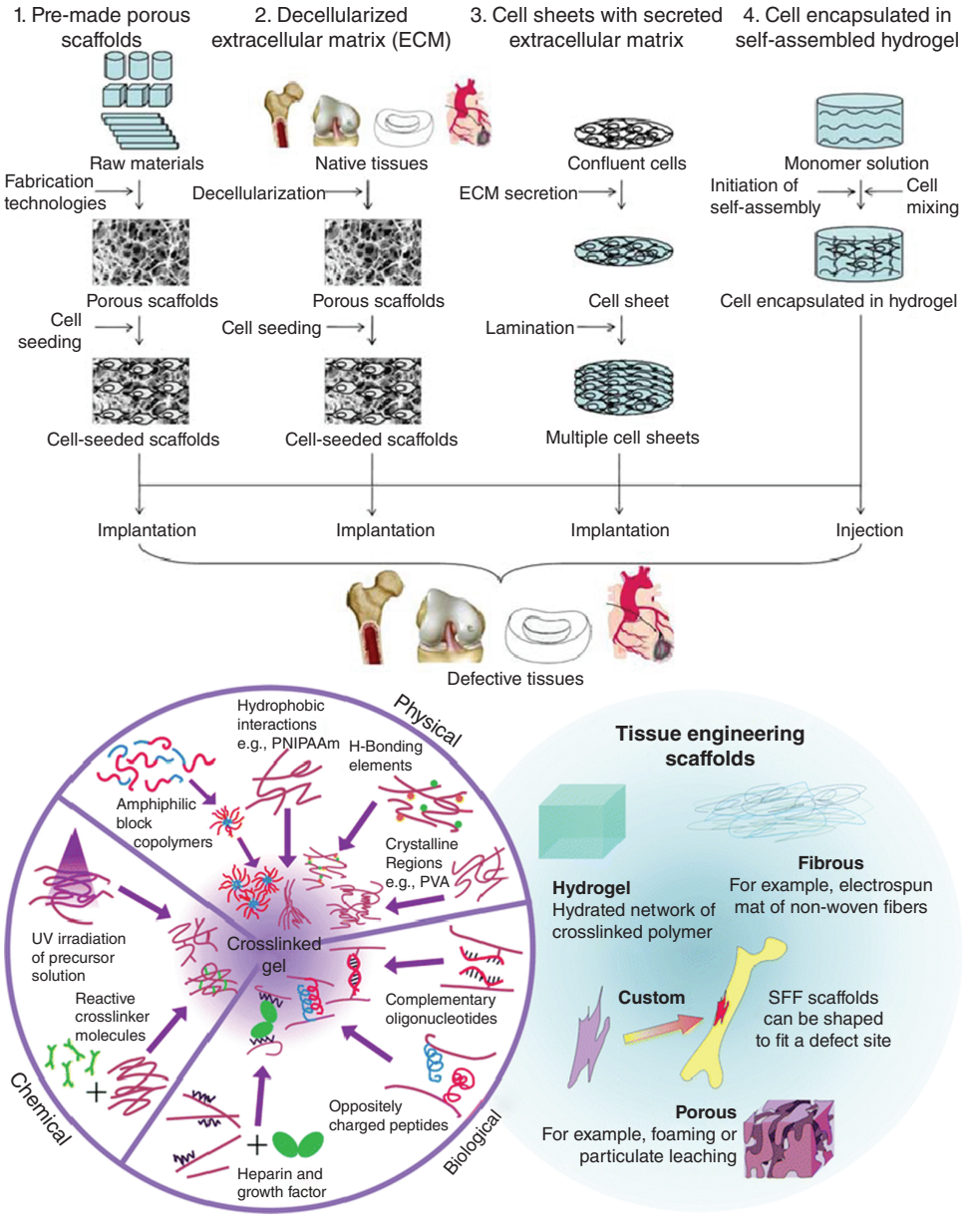

Figure 1.3 Schematic illustration showing different scaffolding approaches that are being used for tissue engineering. These scaffolds can be combined with various physiomechanical strategies to enhance tissue growth.

of regenerative medicine, the injection of a single-cell suspension showed good results replacing a scaffold, but for larger tissue reconstruction more cells are needed, which was the motivation behind cell sheet engineering [26].

1.4.4 Recent Advances in Tissue Engineering

1.4.4.1 Advances in Cell Sourcing and Cell Manipulation

Cells being the building blocks of all living tissues are the starting point for creating tissue substitutes. Growing knowledge about cell manipulation and stem cell differentiation has opened new horizons in the field, providing larger cell pools for all tissue engineering applications. Autologous cells are considered the favorite cell type for engineering tissues, as they do not evoke immune responses and thus eliminate the need for immunosuppressants and their side effects [27]. However, autologous cells are limited in supplies and require a long culture period to engineer the desired tissues. Much of current research aims to use allogeneic [28, 29] or xenogeneic [30] sources to overcome the shortage of autologous cell availability. The use of allogeneic or xenogeneic sources is, though, still associated with major obstacles, such as immune-rejection, transmission of diseases, mismatch between donor and recipient cellular microenvironment, and ethical considerations, which limit their widespread adoption in clinical applications [31].

Applications of stem cells in tissue engineering continue to grow and their use has found its way to the clinic. Although adult mesenchymal stem cells remain the dominant stem cell type used in tissue engineering, embryonic stem cells are also being used and have started to find their way into the market [27]. A major breakthrough in cell sourcing was the recent discovery by Shinya Yamanaka that adult differentiated cells could be induced to become pluripotent stem cells [32]. The discovery of induced pluripotent stem cells (iPSCs), for which Yamanaka was awarded the Nobel Prize in Physiology and Medicine in 2012, has opened unprecedented opportunities in the tissue engineering field by providing a new, large source of autologous cells. A major challenge remains in establishing standardized protocols to induce the differentiation and commitment of differentiated adult, induced, or embryonic stem cells toward the desired lineages.

1.4.4.2 Advances in Biomaterials and Scaffold Production

In the past few decades, a great number of biomaterials from natural and synthetic origins, as well as novel fabrication methods, have been proposed. Current research is focused on developing "smart biomaterials" capable of directing cell functions and/or enhancing cellular performance [33]. The role of the scaffold is to provide structural support and proper signaling cues for cells so that they can replace the scaffold with their own synthesized matrix. Synthesis of new matrix by the cells and degradation of the scaffold should be synchronized so that one process is not faster than the other.

Generally, the goal is to design a scaffold that mimics the structure and composition of the target tissue. Given the complexity of the chemical composition of natural tissues, it is often not possible to fully recapitulate them *in vitro*. Recent developments have led to the establishment of techniques such as bio-printing

[34–37] and two-photon lithography [38, 39], enabling production of precise 3D structures for tissue engineering applications. These techniques also allow the precise positioning of growth factors and recognition sequences for controlled cell behavior [40, 41]. Scaffolds can be prepared with good control over the chemical composition, allowing cells to spread and proliferate (e.g., collagen, gelatin) or inhibiting cell spreading (e.g., alginate, poly(ethylene glycol)). Scaffolds can be made to provide cells with adhesion sequences for cell attachment (e.g., RGD, GFOGER, IKVAV) and matrix metalloproteinase (MMP)-sensitive sequences for scaffold degradation [42–47]. Modifying scaffolds with small molecules, such as phosphate groups and sulfate groups, among others, has been also shown to have strong effects on cell proliferation and stem cell differentiation [48, 49]. All these studies are necessary to identify the ideal scaffolds for each individual tissue engineering application.

1.4.4.3 Advances in Cell Signaling Research and Bioreactor Development

After providing cells with a growing substrate or scaffold, cells require certain signals to survive and synthesize their own matrix that will eventually replace the carrying scaffold. Much knowledge has been acquired about cell signaling, and even more is currently being elucidated. Signals are normally generated by the surrounding cell microenvironment, sensed by receptors on the cell membrane or directly inside the cell, and translated into a variety of cell responses including proliferation, apoptosis, migration, differentiation, and matrix synthesis, among others. The most important signals sensed by cells involve oxygen levels, mechanical stimulation, growth factors, ECM molecules, and other small molecules.

It has been shown, as expected, that different tissues require different combinations of signals, and even the same tissue might require different signals at different depths or different maturation stages. For example, cells used to engineer articular cartilage, which is a relatively simple tissue known to be avascular, require relatively low oxygen levels (below 5%) for the synthesis of type II collagen (the major ECM component of articular cartilage), which in nature is synthesized in high quantities in the deeper cartilage layers. However, to engineer the superficial layer of the tissue, cells require high oxygen levels, which favor synthesis of superficial zone protein (protein mainly synthesized by chondrocytes of the superficial zone and responsible for lubrication) [50]. Moreover, physiologic tensile strain [51] and surface motion [52] are believed to promote superficial zone protein synthesis, while mechanical compression [53] and hydrostatic pressure [54, 55] have been shown to increase type II collagen synthesis (Figure 1.4). Excessive mechanical loading leads to the production of metalloproteinases and aggrecanases that degrade ECM proteins [56]. Systems have been developed for the application of tensile load, compressive load, hydrostatic pressure, shear, and perfusion [53, 57–62]. Notably, stem cells' fate can be steered through the application of phenotypic loading. To illustrate, the application of tensile loads help steer stem cells toward ligament [58], tendon [59], or bone tissue, depending on the tensile load parameters [60, 63], while shear loads can help stem cells differentiate toward cardiac muscle [64] or endothelial cells [61]. Finally, hydrostatic pressure or compression can lead to chondrogenic differentiation [65–68]. The use of bioreactors to

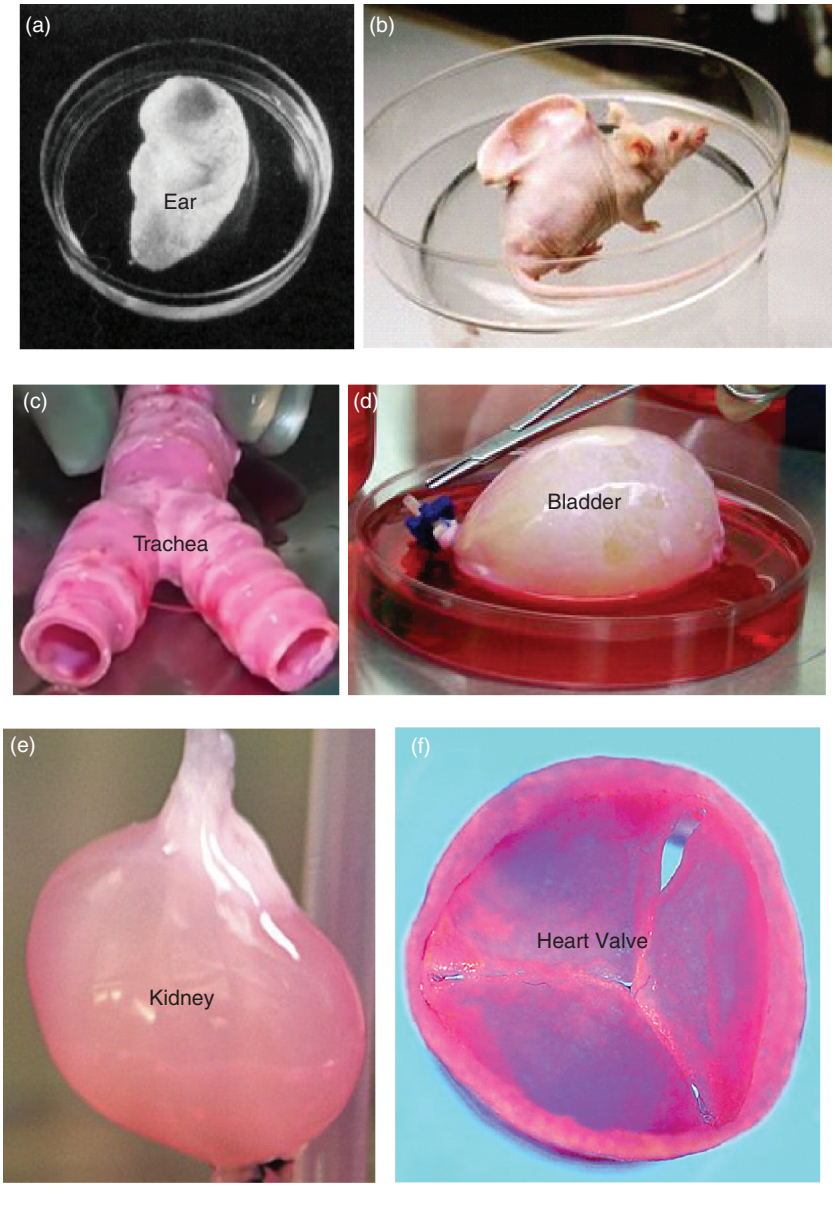

Figure 1.4 Different tissue-engineered organs. (a) Scaffold prepared from synthetic biodegradable polyglycolic acid (PLA) in the shape of a 3-year-old auricle. (b) Scaffolds implanted subcutaneously on the back of an immunodeficient mouse. (Reproduced with permission from [18].) (c) First trachea organ transplant using human's bone marrow stem cells. (d) Constructed artificial bladder seeded with human bladder cells and dipped in a growth solution. (e) Bioengineered kidney that mimics the function of a normal kidney concerning the control of the urinary system and blood filtration. (f) Tissue-engineered heart valve using human marrow stromal cells.

engineer tissues has grown exponentially in the past decade. This is the result of the understanding that tissues need to be subjected to certain forces to adopt a natural phenotype and attain physiologic matrix composition and mechanical integrity. Moreover, bioreactors improve mass transport, which is a prerequisite for engineering 3D complex tissues and organs. Another important role of bioreactors is to standardize, control, and automate the culture conditions to achieve reproducible outcomes. Reproducibility is highly critical in tissue engineering, especially when products might be clinically implemented.

Growth factors, cytokines, ECM molecules, and other small molecules have a profound effect on cell behavior. While some growth factors such as basic fibroblast growth factor (FGF-2) maintain "stemness" of stem cells [69], transforming growth factor beta (TGF-β) induces chondrogenesis [70], bone morphogenic protein (BMP) is necessary for bone formation [63, 71], and nerve growth factor (NGF) is crucial for neural differentiation [72, 73]. In tissue morphogenesis, the production of ECM molecules such as fibronectin and collagens varies depending on the development stage. Changes in the patterns of expression of the ECM molecules are associated with different processes such as stem cell condensation, cell migration, and cell differentiation [74]. Past and current research has revealed much about the function and roles of proteins and genes, but polysaccharides have not been under much focus. Polysaccharides (e.g., hyaluronic acid, chondroitin sulfate, heparin, and heparan sulfate (HS), among others) play a pivotal role in a multitude of physiological and biological processes and possess the ability to encode the function of biological entities analogous to DNA, RNA, and proteins [75, 76]. The sulfation of HS has been implicated in the repair of the central nervous system (CNS). Schwann cells exhibit higher sulfation levels of HS compared to olfactory ensheathing cells during the formation of the gliotic scar. The highly sulfated HS synthesized by Schwann cells is believed to induce a reactive astrocyte phenotype, which inhibits axon growth following CNS injuries [77]. This information can be used to improve current treatments of neural injuries or to design better neural tissue engineering products.

1.4.4.4 Engineering Complex Tissues and Organs

Tissue engineering holds strong promise of providing substitutes for damaged tissues and organs. However, the field that is less than 50 years old can be considered to be still in its infancy. Tissue engineering has found initial success with the production of simple tissues such as skin [5, 78] and cartilage [22]. Over the past few years, more complex multicellular tissues and organs have been engineered, including urethras [79], tracheas [80], blood vessels [81, 82], airways [85], and bladders [9, 86]. Advances in the tissue engineering field have also reflected in high economic returns for tissue engineering, which grew from $7.5 billion in 2010 to about $15 billion in 2014 and is expected to reach $32 billion by 2018 [10]. So far, tissues were mainly engineered using membranes with one cell type cultured on each side; therefore, they were based on 2D culture techniques. However, engineering of more complex 3D tissues is still limited by several factors affected by all elements of the tissue engineering triad. The most important challenge facing the development of 3D complex tissues

is mass transport that governs access of nutrients and secretion of wastes in engineered tissues [87, 88]. Circulation of nutrients and wastes in natural tissues *in vivo* is controlled by blood vessels. In tissue-engineered structures, mass transport can be achieved by using bioreactors, as mentioned previously, or by inducing the formation of new blood vessels. Efforts have focused on developing scaffolds with certain patterns or coatings to induce neovascularization, cell manipulation to induce differentiation, or secretion of vascular endothelial growth factors (VEGF) and proper signaling such as the addition of growth factors.

1.5 Applications of Tissue Engineering

Tissue engineering is a young field that utilizes cells, biomaterials, physical signals (e.g., mechanical stimulation), biochemical signals (e.g., growth factors and cytokines), and their combinations, to engineer tissues. The most common application of tissue engineering is to create tissues that can be used to repair or replace tissues in the body suffering partial or complete loss of function. However, tissue engineering has started to find new applications such as the development of extracorporeal life support units (e.g., bioartificial liver and kidney), *in vitro* disease models, tissues for drug screening, smart diagnosis, and personalized medicine. These applications will be discussed in more details in the following sections. Figure 1.5 depicts some of these applications.

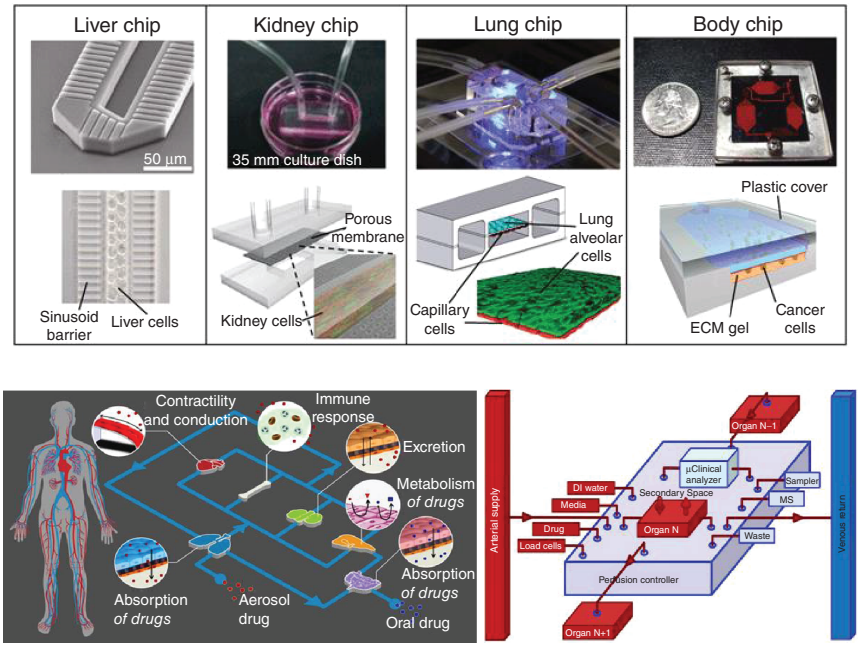

Figure 1.5 Recent advances in the applications of microfluidics in developing "organs-on-chips" models for *in vitro* investigations of engineered tissues and organs.

1.5.1 Implantable Tissues and Organs

Tissue engineering came to being as a solution to the partial or complete loss of organ functions due to congenital failure, disease, or injury. Tissue engineering may be used to restore different tissues including connective (e.g., bone, cartilage, blood), muscle (e.g., cardiac, skeletal), epithelial (e.g., skin, linings of the digestive tract), and nervous tissues (e.g., central nervous tissue, peripheral nervous tissues).

Skin epidermal tissue was the first tissue to be investigated for the purpose of skin replacement [89]. The earliest attempts to grow Keratinocytes *in vitro* were based on explant or organ cultures, which soon appeared to be overgrown with fibroblasts and exhibited limited proliferation [90, 91]. The discovery of Puck *et al*. [92] in 1956 that lethally irradiated epithelial cells can provide mitogens without proliferation opened the way for the use of these cells as a feeder layer for a cocultured cell layer. The feeder layer concept was used in 1975 by Green and Rheinwatd to grow human epithelial cells, leading to the first product of tissue engineering [3, 4]. In their work, Green and coworkers describe methods to grow skin epidermis using a skin biopsy from the patient. The harvested biopsy is digested to retrieve autologous keratinocytes, which are then cocultured with a feeder layer of mouse mesenchymal stem cells for several weeks to reach sufficient cell numbers. This approach was then commercialized under the name "Epicel" to produce autologous sheets of keratinocytes used to treat patients suffering from burn accidents. Subsequent research focused on improving the culture medium by adding calcium and hormones, enabling the growth of stratified keratinocyte sheets that do not require feeder layers. However, these sheets are very fragile and prone to damage at any stage from the laboratory to the patient. Therefore, large efforts have been made to overcome stability issues, such as the use of polyurethane backing materials and, more recently, the use of aerosol systems to spray keratinocytes directly onto the wound [93, 94].

Another tissue that was under focus in tissue engineering was cartilage. The first attempts to use cell-based techniques to repair cartilage defects were made by Peterson and coworkers in the late 1980s [95]. The technique later known as *autologous chondrocyte transplantation* (ACT) was described in humans by Brittberg *et al.* in 1994 [96]. In ACT, chondrocytes are isolated from a biopsy of healthy autologous cartilage and expanded *in vitro* for several weeks to reach sufficient numbers. A periosteal flap large enough to cover the lesion is harvested from the proximal medial tibia and sutured to the cartilage surface leaving a small gap for cell injection. The spaces between the sutures are filled with fibrin glue to prevent cell leakage. The injected chondrocytes are then expected to form a new cartilage that will be able to integrate with the surrounding tissue and withstand daily loads [97–99]. Although ACT presented a major advancement in cartilage therapy, periosteal hypertrophy encouraged the research for improved repair strategies [100, 101]. In order to address the above problem, a membrane based on type I/III porcine collagen has been developed and is used to replace the periosteal flap in ACT procedures [102–104]. The membrane, commercialized as ChondroGide®, has a porous surface on the side that faces the defect, which

allows cell attachment, and a smooth compact surface that prevents cell leakage. Although ACT and its variants represented a major step in cartilage therapy, they still face several challenges. The most pronounced challenge is the loss of the cartilage phenotype during *in vitro* monolayer expansion, known as *chondrocyte dedifferentiation* [105]. Chondrocyte dedifferentiation is associated with morphological and gene expression changes where cells behave more as fibroblasts [106, 107]. Dedifferentiated chondrocytes produce type I collagen rather type II collagen, and thus cells implanted in ACT procedures often produce fibrous tissue and not hyaline cartilage [108]. Today's research focuses on developing a variety of methods to either prevent dedifferentiation during serial expansion or to restore the cartilage phenotype of cells before or after their delivery to the defect site [109, 110]. Another challenge is to reproduce the stratified cartilage structure that is formed of structurally and chemically distinct layers at the level of cells and matrix. This structure helps the cartilage to better withstand and respond to mechanical stresses. Except for very few studies, cartilage has been traditionally treated by tissue engineers as a single layered tissue, which is due to the fact that cartilage contains only one cell type (chondrocytes). A very critical component in the engineering of cartilage tissue is to select the proper scaffold, which can be made of synthetic or natural materials and may have different mechanical, chemical, and physical properties. Another important component of tissue engineering is signals (mechanical or chemical). Currently available tissue engineering strategies for cartilage repair do not yet fully recapitulate the cartilage ultrastructure or the cartilage microenvironment that provides a multitude of cues necessary for cartilage homeostasis [111]. In its original form, ACT does not use all elements of tissue engineering, especially the scaffold and signaling (chemical and physical). So far, numerous scaffolds have been proposed to be used as chondrocyte carriers in ACT-like procedures. Additionally, chemical and mechanical signaling was used either before or after tissue transplantation. Moreover, various cell sources have been investigated, including chondrocytes, mesenchymal stem cells, adipose-derived stem cells, and, most recently, iPSCs. Although each of these systems has its advantages, there is not yet a consensus that verdicts one of these approaches as the ultimate cartilage tissue engineering approach. Current cartilage tissue engineering research is focused on understanding basic questions about cells, their interaction with their matrix, and response to mechanical stimulation in health and disease to produce an ideal engineered cartilage. The knowledge gained in the coming years in the tissue engineering triad will enable the engineering of chemically and mechanically functional cartilage.

In addition to skin and cartilage, tissue engineering and regenerative medicine has made significant steps toward bone repair. Bone tissue naturally remodels and may repair itself in case of small fractures. However, natural repair does not often occur with severe bone injuries such as non-union fractures or when extensive bone removal is performed in case of malignancy, infections, and reconstructive operations. In these cases, bone grafting is performed mainly using autologous grafts, but also possibly using allograft and xenografts. To overcome possible immunogenicity from the use of nonhuman grafts, bone extracts have been proposed, such as the use of demineralized bone matrix (DBM) [112]. Despite the

various protocols applied in the preparation of nonhuman grafts, immunogenicity remains an issue. Apart from the use of bone tissue and DBM, collagen and porous ceramics including phosphate- and calcium-based ceramics have been also employed [113]. Nevertheless, the best repair is usually observed with autografts, which, however, present some critical drawbacks such as the limited availability, surgery complications, donor site morbidity, and pain. The limitations associated with the use of autografts encourage the search for alternatives, of which tissue engineering might be the most ideal.

Tissue engineering and regenerative medicine strategies used for bone repair follow the basic tissue engineering methods and therefore rely on the combination of scaffolds, cells, and signals. The engineered implant should support one or more of the following properties: osteoconduction (implant allows good integration with host tissue and bone spreading), osteoinduction (implant encourages bone formation by inducing cell differentiation toward the bone lineage, e.g., DBM and uroepithelium) [114–116], or osteogenesis (bone formation by specific osteoprogenitors) [117]. The main role of scaffolds in bone tissue engineering and regenerative medicine is to serve as a mechanical support that structurally fills the defective bone area. This silent mechanical role can be improved by adding biologically/chemically active components that further enhance or accelerate bone repair (e.g., cells, growth factors, enzymes, and attachment moieties). The success of the scaffold relies on a number of parameters, including mechanical properties (e.g., compressive modulus), structural properties (e.g., porosity), biocompatibility (does not evoke toxic or inflammatory reactions), and biodegradability (scaffold degrades slowly to be replaced by newly formed bone). Scaffolds that mimic the inorganic bone component such as tricalcium phosphate (TCP) and hydroxyapatite (HA) are biocompatible, with the former being highly biodegradable and the latter being nondegradable. Many other biodegradable scaffolds have been investigated for bone tissue engineering, including poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(DL-lactic-*co*-glycolic acid) (PLGA), and poly(ε-caprolactone) (PCL). Additionally, polymers such as polyorthoester (POE), polyanhydrides, and poly(propylene fumarate) (PPF) have shown good biocompatibility in animal models [113, 118]. The biocompatibility of bone grafts can be improved by modification with PEG, while biodegradability can be increased by incorporating MMP-sensitive peptides. The modification of scaffolds with ECM molecules (e.g., collagen) or peptides (e.g., Arg-Gly-Asp (RGD), and more recently Gly-Phe-Hyp-Gly-Glu-Arg (GFOGER)) improve cell attachment and consequently enhance osteoconductivity. A second important pillar in bone tissue engineering and regenerative medicine is growth factors, which play fundamental roles in bone repair and bone formation. BMPs, which comprise over 20 different isoforms, have been shown to induce bone formation especially BMP-2 and BMP-7 [119, 120]. In addition to BMPs, other growth factors such as TGF-β [121], VEGF [122], fibroblastic growth factor (FGF), insulin-like growth factor (IGF), and platelet-derived growth factor (PDGF) are involved in bone repair and bone formation [123]. Finally, cells can be included in bone tissue engineering strategies to increase the healing efficiency of the implant or to initiate bone formation *in vitro* prior to implantation. Bone marrow stromal

cells (BMSCs) are the most commonly used types, which are able to differentiate toward the osteogenic lineage [63, 117, 124]. However, other cells have also been shown to possess the potential to form bone-like tissue such as adipose-derived stem cells [125], muscle-derived [126], dermal-derived [127], placenta-derived stem cells [128], embryonic stem cells [129], and recently induced pluripotent stem cells [130]. The combination of some or all the pillars of tissue engineering described above could lead to the successful bone formation.

TERM has made significant steps toward the repair of almost every human tissue. Peripheral nerve injuries and spinal cord injuries can be treated using silicon- or collagen-based nerve guides (hollow tubes), which can be combined with matrices, scaffolds, growth factors, and/or cells to improve repair. In urogenital tissues, engineering the bladder has been achieved with a good degree of success in dogs by seeding urothelial cells and smooth muscle cells on acellular matrices [131]. Atala and coworkers took the procedure one step further by seeding autologous epithelial and smooth muscle cells on biodegradable collagen or collagen/PGA bladder-shaped scaffolds and implanting them in patients with myelomeningocele with high pressure or poorly compliant bladders [86]. Urethral tissue has been engineered and clinically tested in animal models using a strategy similar to the above, where autologous epithelial and smooth muscle cells isolated from a bladder biopsy were seeded in the lumen and outer surface, respectively, of a collagen matrix [132]. Atala and coworkers used similar techniques to engineer uterus and vaginal tissues as well [133]. Attempts towards engineering a kidney – the first organ to be transplanted – have also been made despite the complexity of the tissue. Renal tissue can be engineered by growing renal cells on tubular polycarbonate membranes to be used as extracorporeal dialysis units or to be implanted to replace injured kidneys [134]. Testicle tissue engineering has been attempted, where Leydig cells were encapsulated in alginate–poly-L-lysine spheres, and shown to maintain normal testosterone levels [135]. Penile tissues such as corporal tissue have been engineered using autologous rabbit collagen matrices seeded with autologous smooth muscle cells and endothelial cells. The engineered corporal tissue was implanted in experimental rabbits, which enabled normal erection, mating, and conceiving [136]. Liver tissues have been engineered by combining hepatocytes with PGA/PLGA scaffolds, and vascularization was supplied either by using a vascular bed or using porous scaffolds that allow angiogenesis [137]. Bioartificial patches for tracheal replacement have been developed by seeding autologous muscle cells and fibroblast cells on porcine collagen matrices. The patch was able to create an airtight cover for a tracheal opening, enabling neovascularization, and was covered with viable ciliated respiratory epithelium [85, 138]. Pancreas tissue engineering involves mostly employing islet β-cells or insulin-producing cells (e.g., differentiated stem cells, progenitors, or genetically engineered somatic cells) delivered alone or within a matrix such as calcium alginate/poly-L-lysine/alginate (APA) beads for immunoprotection [139]. Finally, TERM of bowel tissues including the intestines and the stomach has witnessed significant advancements. In 2003, Vacanti and coworkers engineered intestinal tissue by seeding cells harvested from intestinal organoids on a tubular-shaped collagen-coated PGA scaffold [140, 141]. The engineered

structures were implanted in the omenta of animals and exhibited several normal intestine characteristics (e.g., epithelium submucosa and muscular layers) in addition to good angiogenesis from omental vessels. Different biomaterials have been used for gut tissue engineering applications, including synthetic biomaterials (e.g., PGA, PLGA, and PLA), natural materials (e.g., collagen, fibrin), and acellular scaffolds (e.g., small intestine submucosa). Scaffolds can be engineered to release specific growth factors relevant for intestinal development. Moreover, different cell sources have been investigated, including stem cells and genetically modified cells (Rocha and Whang, 2004).

Furthermore, extensive research is being done on cardiac tissue engineering. It aims to create functional tissue constructs that can reestablish the structure and function of injured myocardium.

1.5.2 *In Vitro* Models for Disease Studies

While most currently available engineered tissues are used for restoring organ functions in the body, new applications are currently being considered for using these tissues in disease models. The aim of this technique is to be able to control and ideally to cure many diseases that are still incurable. To test the effects of certain pharmaceuticals, scientists have been using animal models with some gene alterations to represent the human diseases. While this approach has been highly useful, some mechanisms can actually differ between animals and humans. With the impossibility of direct testing on humans and the failure of simple human cell cultures to mimic the disease behavior at the organ or tissue level, tissue engineering came in as the best option to model human diseases [142].

Tissue engineering for disease models aims to mimic the natural properties available *in vivo* such as architecture, environment, growth factors, and biomechanics [143]. In this case, the tissue is just an intermediate step toward the development of the actual treatment. This is why it is usually simple and small in size to minimize the regulatory requirements such as oxygen supply [144]. That being said, a variety of methods are used to produce the required tissues for disease models; these methods combine stem cell biology and the recent advances in tissue engineering, such as the use of scaffolds, bioreactors, organ-on-chip systems, and even 3D printing technologies [142]. The result can be a 2D network of neural cells from human iPSCs, a 3D structure of complex organs like heart valves based on valvular endothelial cells, or organ-on-chip models that rely on microchip manufacturing technology.

Although this approach is still in its earliest stages, many successful usages of engineered tissues for modeling diseases have been noted already. The first example is that of skin equivalents (SE), which have been developed as models that mimic the human skin. These engineered tissues have been useful to study the normal and altered behavior of the skin. They started off as a collagen matrix with dermal fibroblasts inside, and then were subject to more optimization so they would be able to mimic the actual *in vivo* characteristics. The new models were suitable to study many human disease processes such as the skin response to early cancer development or to wound healing [145].

Heart diseases are also being modeled through tissue constructs of human myocardium. These tissues are recently being produced using cardiomyocytes derived from human embryonic stem cells and from human iPSCs; and among the different heart diseases that are modeled are myocardial fibrosis, cryoinjury-induced myocardial infarction, dilated cardiomyopathy, and LEOPARD syndrome [146].

Other than skin and heart diseases, many others are also being modeled. For example, an *in vitro* model of Parkinson's disease has been created using a microfluidic channel with a concentration gradient of neurotoxins [147]. Moreover, engineered tissues of the liver are currently being developed to model liver diseases and try to find new treatment modalities [148]. Lungs, cartilage, intestine, kidney, bone marrow, and vascular diseases also have their share in tissue engineering models, as well as those related to the endocrine and nervous systems. Even cancer and infectious diseases are increasingly being modeled through 3D tissues, although the field is still in its infancy. For a more comprehensive review of these various models, we refer the reader to [142].

It is good to mention that, despite the huge progress that is happening within the field of disease modeling, it is still very complex to model human diseases with their complexity; this is why instead of aiming to solve the problem in its complexity, scientists are focusing more on simpler models that can replicate the basic structure and function of tissues, before dealing with more complex models of organs and systems [142].

1.5.3 Smart Diagnosis and Personalized Medicine

Personalized medicine is a novel approach that takes into consideration the unique characteristics of each patient and his or her individual response to various drugs. While it is still an emerging area of scientific investigations, it is very likely that it will govern the future of medicine [149].

The development of tissue engineering has helped the advancement of this field on many levels, the first being related to drug testing. In fact, since a 3D model of a patient's organ can be engineered by seeding the person's cells into a scaffold, it is possible to test the efficacy of different treatments on it. The human body utilizes many drug-metabolizing enzymes and drug transporters to deal with the drugs in the body. An example is cytochrome P450 (CYP), which participates in the metabolic process of many drugs. Since the CYPs genotype variation is thought to affect the individual's response to a particular drug, it would be helpful to use the engineered tissues to test the drugs for different patients [150].

Another way tissue engineering can pave the way for personalized medicine is through tailoring the tissue construct itself to fit the needs of a specific patient. For the artificial scaffold to be properly functional, it should not only allow the cells' survival but it should also be compatible with the cells' microenvironment and with the host tissue's mechanical, physical, and chemical properties. Personalized therapy has been used, for example, in tissue engineering of urethras using the patients' own cells. Because these scaffolds were compatible with the body

they were implanted in, the grafts ended up developing a normal architecture and a proper functioning [151].

Another promising application of tissue engineering is in the diagnostics field. Instead of using the traditional medical imaging techniques to provide information about the patients' internal organs, sometimes having a physical prototype is more useful. Using manufacturing techniques and rapid prototyping, many studies have created anatomical models replicating organs and tissues to make testing easier. On the other hand, the development of lab-on-a-chip devices as micro-engineered tissues has made testing procedures such as extracting blood or DNA samples much more effective [152].

1.6 Challenges in Tissue Engineering

Despite all the advances in the field of tissue engineering, many challenges persist, which are related to three elements of cells, scaffold, and signals. Starting with the cells, the sources to get them and then seed them in the scaffold are numerous. In fact, autologous, allogeneic, and xenogeneic cells are all potential sources, and each of these can be subdivided into stem cells (adult or embryonic) or differentiated cells. Since they all have their own advantages and disadvantages (immune reaction, differentiation, etc.), the choice of the right source for the cells and their culture is a challenge by itself [153].

The choice of scaffold biomaterials is not an easier task either. The scaffolds must actually respond to both the structural and functional requirements of the body. It must be biocompatible and should be able to communicate with the ECM while at the same time providing the needed mechanical support [154]. While natural materials have better biocompatibility and biodegradability, synthetic ones usually present stronger mechanical properties. This is why the use of composite materials is sometimes required, which also allows the scaffold to have its required porous structure [155].

Another important challenge in tissue engineering is related to the transportation of nutrients and waste secretion in the engineered tissue [87]. Since the majority of tissues rely on blood vessels to transport oxygen and nutrients, the 3D engineered tissue needs to be vascularized with a vascular capillary network [88]. This is not an easy task; after the implantation of the scaffold inside the body, the oxygen available is directly consumed and new vessels are formed only after several days [153]. Alternative methods to angiogenesis are thus necessary, and many techniques for prevascularization of the engineered tissues have been suggested based on subtractive, additive, and hybrid methods [156].

Finally, a major challenge is still present, namely mass production and commercialization of the engineered tissues. Specific manufacturing conditions and quality control strategies need to be ensured. In addition, answering the exact needs of the patients (demand) and providing long-term storage and shipping facilities while ensuring that the structure and function of the tissues are intact are also of great importance [154].

1.7 The Future of Tissue Engineering

The last few decades have witnessed major steps in health care, leading to improved surgical procedures and better management of diseases. All in all, the advances in the health care have raised life expectancy, augmenting vulnerability to diseases and organ failure. Consequently, the aforementioned advancements have led to an increased demand for tissues and organs. The ultimate goal of tissue engineering is to bridge the constantly growing gap between organ demand and availability by producing complete organs [157]. This area is expected to become increasingly applied as a valid clinical solution.

Stem cells will continue to be investigated for their differentiation potential, and more applications will be developed in the future. The major challenge for stem cells, whether induced, embryonic, or adult, is to achieve commitment to the desired lineages. It is expected that more applications using stem cells will reach clinical trials in the near future. Furthermore, gene therapy (silencing and activation of target genes) and drug delivery are both expected to be used to help maintain the desired cell phenotype. The ultimate goal would be to engineer immune-transparent stem-like cells with clear protocols, enabling their committed differentiation to targeted tissues. Developments in basic and applied science related to the fabrication of tissue engineering scaffolds will be a major future target. High-throughput screening techniques might prove useful to determine combinatorial effects of molecules and materials on various cell types. Decellularized tissues are also expected to remain an important source of scaffolds given their high abundance as well as their right chemical and structural composition. Potential limitations of such scaffolds will always be the shortage of supplies (e.g., scaffolds from allogeneic sources), potential immunoreactions, and ethical concerns (e.g., scaffolds from xenogeneic origins). In future, it is expected that new biomaterials will be developed incorporating selected molecules to address targeted tissues. Moreover, many basic science studies will be conducted to identify the effects of molecules on cells and determine the right degradation rate and material properties (porosity, mechanical properties, and structural properties) suitable for each tissue engineering application. An ultimate goal would be to combine scaffolds and cells to engineer tissues *in vitro*, which can be decellularized to produce customizable off-the-shelf tissue sources for various engineering applications. Future research will continue to reveal the roles of ECM molecules to define ideal recipes to engineer constructs that most closely resemble natural tissues.

The mechanisms through which cells perceive load and react to their surrounding environment are only starting to be revealed and comprise stretch-activated ion channels and integrins [158, 159]. Understanding these mechanisms will provide the basis for developing new tissue engineering tools and bioreactors, and possibly discovering new useful molecules for the treatment of sick organs and tissues. Future bioreactors will be able to perform complex combinatorial tasks in order to engineer full organs. For example, bioreactors can be designed to deliver varying oxygen levels to varying parts of the engineered tissue or different mechanical stimulation regimes, or to deliver growth factors and molecules

at predefined time points during culture. Finally, bioreactors may be made to be used on site (e.g., in the hospital) to minimize contamination risks and reduce the surgery time.

1.8 Conclusions

The field of tissue engineering has witnessed tremendous development in the past few decades, which has brought to the clinics solutions once believed to fall under science fiction. Although the application of tissue engineering principles is not widespread in clinics, a very bright future is expected for the field where more tissues will join the list of "clinically applicable tissue engineered constructs." A combination of immune-transparent cells with an off-the-shelf scaffold cultured in a complex bioreactor that delivers tailored signals for the target tissue is probably expected to become possible in the future. However, reaching the stage of clinically relevant off-the-shelf body parts still requires significant basic and applied scientific research.

Future efforts will focus on developing novel biomaterials for the different tissue engineering and regenerative medicine applications. The structure and mechanical properties of the biomaterials will be engineered to better suit the tissue of interest. These biomaterials should be capable of addressing the current major limitations of the field, especially mass transport. Moreover, the developed biomaterials are expected to be better tailored to maintain the phenotype of cultured cells and deliver on demand the optimal cocktail of growth factors and cytokines. Research should also focus on materials that would reduce implant complexity such as injectability or flexibility that allows minimally invasive surgical procedures. Finally, materials that have better integration or stability in the implant site should be designed. Biomaterials with muscle-adhesive proteins and other gluing interfaces may be investigated, or using covalent bonding based on natural residues of tissues and engineered residues on the scaffold. Future research will also focus on cell manipulation (e.g., transfection and silencing) to induce better repair or regeneration. Further understanding at the basic science level of cell behavior, both *in vitro* and *in vivo*, in tissue engineering systems including cell–cell interactions and cell–scaffold interactions will be required. Additionally, the effect of different growth factors as well as ideal amounts and timing of supplementation should be determined for the various tissue engineering applications. *In vitro* culture techniques should also be revised, particularly the switch from 2D to 3D systems and oxygen levels to match the *in vivo* situation of thick tissues. Perhaps, systematic studies that compare current *in vitro* culture systems used in tissue engineering and the *in vivo* situation will shed light on the biological effects of the currently adopted culturing techniques. This knowledge can be used to improve current cell culture techniques to achieve better tissue repair. Finally, efforts should be made toward optimizing current regulatory and ethical considerations that would pave the way for easier and safer introduction of tissue engineering and regenerative medicine solutions into the clinic.

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