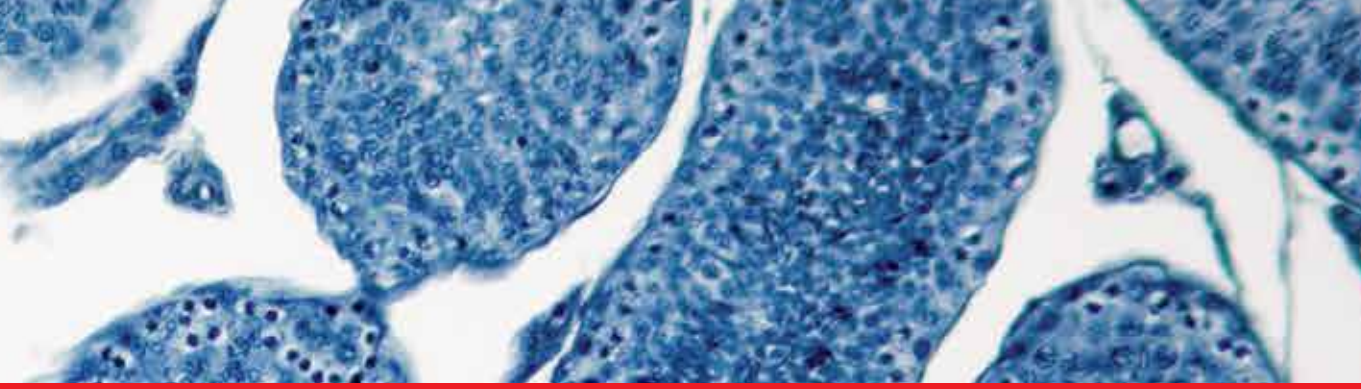

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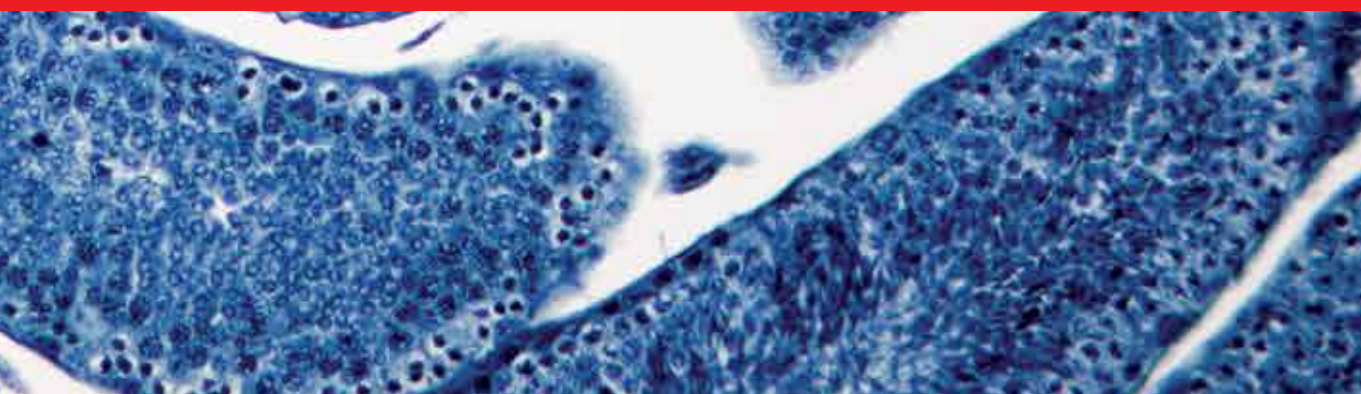
Tissue Regeneration

Edited by Hussein Abdel hay El-Sayed Kaoud



TISSUE REGENERATION

Edited by **Hussein Abdel hay El-Sayed Kaoud**

Tissue Regeneration
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Meet the editor

Dr. Hussein Kaoud was the Chairman of the Department of Preventive Medicine of the Cairo University. He has given lectures on Molecular Epidemiology and Biotechnology at different universities. He is a member of many international publishing houses and a reviewer and an editor of indexed journals.

Currently, he is working as a full Professor of Preventive Medicine at the Cairo University, Egypt. His research interest focused on molecular biology and advanced technology of basic life sciences after he received his PhD and DSc degrees.

He has published more than 265 publications and has several international books. He received 1 International Award (USA), 10 Cairo University International Publication Awards, and the Appreciation Award in Advanced Technological Sciences from the Cairo University. He supervised, examined and discussed many medical dissertations.

Contents

Preface

Tissue regeneration is a vast subject, with many different important aspects to consider. Regenerative medicine is a new branch of medicine that tries to change the course of chronic diseases and, in many cases, regenerates the organ systems that fail due to age, disease, damage or genetic defects.

The main purpose of this book is to point out the interest of some important topics of tissue regeneration and the progress in this field as well as the variety of different surgical fields and operations. This book includes 7 sections and 11 chapters that provide an overview of the essentials in tissue regeneration science and their potential applications in surgery.

The authors of each chapter have given consolidated information on ground realities and attempted to provide a comprehensive knowledge of tissue engineering and regeneration. This book will be useful to researchers and students of biological and biomedical sciences (medical and veterinarian researchers).

This book is the result of the collaborating parties. I gratefully acknowledge the assistance provided by all the authors who have contributed to the publication of this volume and by the IntechOpen editorial office that initiated this project and has completed the book edition.

The editor is thankful to every individual who helped in the preparation of this book. The editor is also indebted to the chapter contributors for accepting helpful criticism for the present shape of the book.

Finally, my special thanks go to Ms. Lada Bozic, Author Service Manager at IntechOpen, for sending information and guidelines for editing the book chapters well on time.

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Introduction

Introductory Chapter: Concepts of Tissue Regeneration

Hussein Abdelhay El-Sayed Kaoud

Additional information is available at the end of the chapter

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1. Biology of regeneration

Regeneration means the re-growth of part of the affected or lost organs of the remaining tissue. Animals can regenerate some organs, such as the liver. If a part of the liver is lost due to illness or injury, the liver grows back to its original size, but not in its original form.

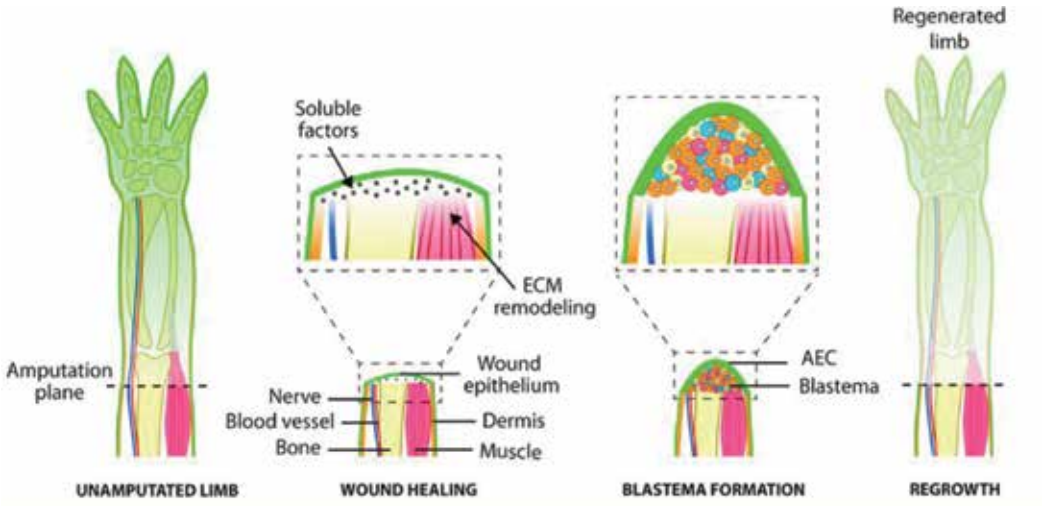

Figure 1. The amphibian renewal preceding amphibian growth may offer hints to humans. After amputation, the wound heals to form the skin layer, and the underlying tissue undergoes a matrix reshaping, and cells in the region secrete soluble factors. The heterogeneous cell mass, blastema, is formed by the proliferation and migration of cells from neighboring tissues. Next, the blastema leads to the appearance of different new tissues that are spatially plastered to reconstruct the structure of the original limb (credit: Lina et al. [1]).

Different species have significant capabilities to regenerate parts of the body or whole organism after injury (**Figure 1**), but a thorough understanding of the molecular basis of regeneration mechanisms will require detailed genomic resources.

2. Building blocks and matrix

2.1. Cells

Cells are building units of tissues and organs and tissues are the basic unit of function in the body. In general, cells secrete their own support materials and structures, which are called an *extracellular matrix*. This matrix, or scaffold, is supporting the cells; it also performs as a relay station for a number signaling molecules [2].

2.2. Messages/signals

Cells acquire messages through multiple sources that grow available from the local environment. Each signal can enhance or initiate a series of responses that decide what will happen to the cell. By understanding how individual cells react to signals, interact with their surrounding environment, and organize them into tissues and organisms, researchers can manipulate these processes to repair damaged tissue or even create new cells.

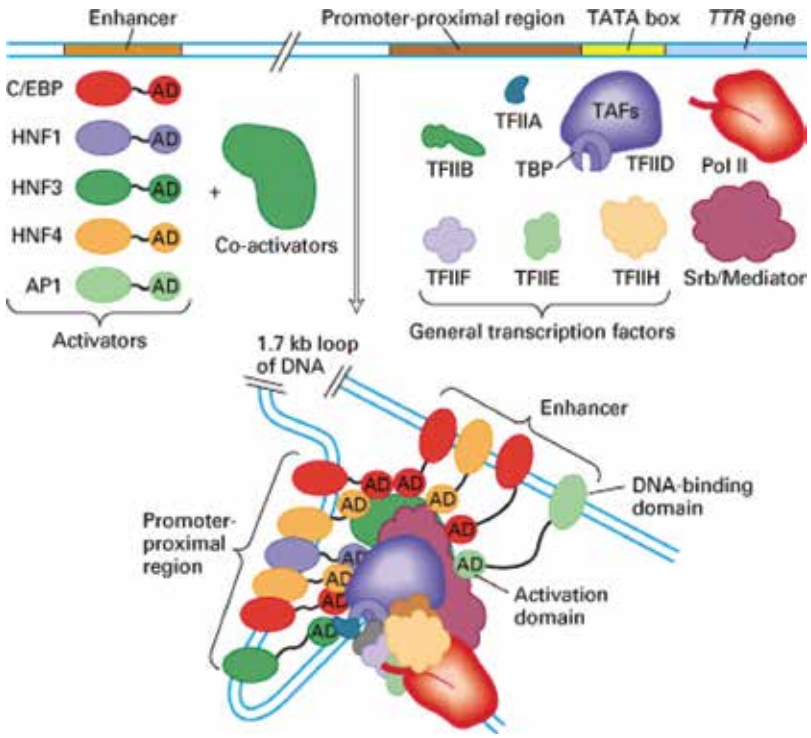

Figure 2. A view of a eukaryotic gene, its control elements in the DNA and the proteins that guide the RNA polymerase to the correct starting point for transcription [3].

In all organisms, a *DNA-dependent RNA polymerase* is performing the production of mRNA for protein synthesis or the various non-coding RNA molecules that are used in the cell. *Transcriptional control* is the main method to control what proteins (and nucleic acids) are produced in the cell, and in what amounts (**Figure 2**).

3. Stem cells and regeneration

Tissues in the human organism are generated, maintained, and repopulated by *stem cells* (**Figure 3**). These are specialized cells capable of cell renewal and can differentiate into different cell types in the human body. Stem cells have several differentiation programs; therefore, they possess information to allow them to become any cell in the body or a restricted cell type with a specialized function. These abilities make stem cells extremely useful for biomedical applications and regenerative medicine and have become the main molecular tool for these purposes. Skeletal muscles have some ability to regenerate and form new muscle tissue, while cardiac muscle cells do not regenerate. However, new research suggests that cardiac stem cells may be coaxed into regenerating cardiac muscles with new medical strategies. Smooth muscle cells have the greatest ability to regenerate.

Questions about how and why tissue regeneration attracts the attention of countless biologists, medical engineers, and doctors. Renewable capacity varies widely across organs and organisms and a range of model systems with different technical features and innovation strategies are studied. Several key issues common to natural regeneration are receiving new attention from improved models and approaches, including identification of innovative

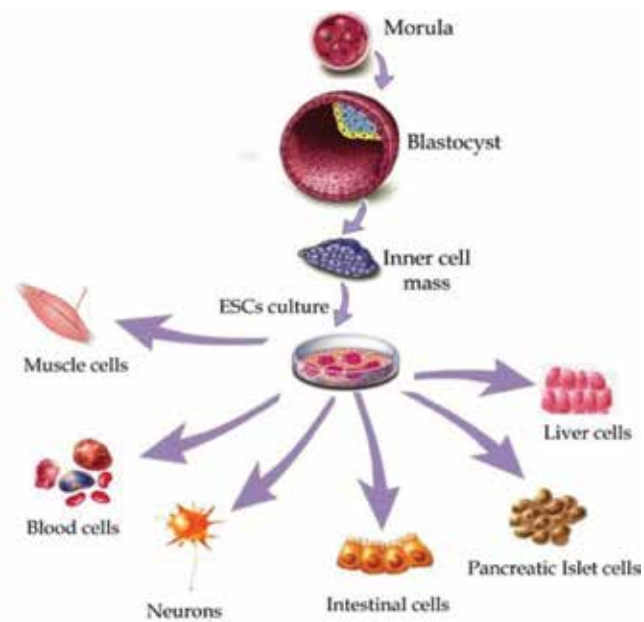

Figure 3. Induced pluripotent stem cells (iPSCs) were first created from human cells in 2007. These are adult cells that have been genetically converted to an embryonic stem cell-like state [3].

capacity; importance of stem cells, differentiation and differentiation; how regeneration signals begin and target; and mechanisms that control proliferation and renewed regeneration.

4. Regenerative medicine

Regenerative medicine is a new branch of medicine that tries to change the course of chronic diseases, and in many cases, regenerates organ systems that fail due to age, disease, damage, or genetic defects. The area has quickly become one of the promising treatment options for patients with tissue failure. It also includes tissue engineering, but also involves the search for self-healing—the body uses its own systems, sometimes with the help of foreign biological materials to reconstitute cells and rebuild tissues and organs. The terms "*tissue engineering*" and "*regenerative medicine*" have become highly interchangeable, with the field hoping to focus on treatments rather than complex and often chronic disease treatments.

Tissue engineering is an emerging biomedical field aimed at helping to restore physical tissue defects to the point of self-repair as well as replacing the biological functions of damaged and injured members using cells with reproductive and differential abilities. In addition to basic research on these cells, there is no doubt that successful tissue engineering is indispensable for creating an artificial environment that enables cells to stimulate tissue regeneration. Such an environment can be achieved using scaffolds for cell proliferation, differentiation, and growth factors, as well as combining them. Growth factors are often required to promote tissue regeneration, as they can stimulate the formation of blood vessels, which supply oxygen and nutrients to the transplanted cells to replace the organ to maintain its biological functions.

It requires functional platforms or scaffolds with specific properties concerning the morphology, chemistry of the surface, and interconnectivity to promote cell adhesion and proliferation. These requisites are not only important for cellular migration but also to supply nutrients and expulsion of waste molecules. Cell type must be considered when designing of using a specific cellular grown system as scaffold; for instance, if they are autologous, allogeneic, or xenogeneic. The challenge in tissue engineering is to develop an organized three-dimensional architecture with functional characteristics that mimic the extracellular matrix. In this regard, with the advent of nanotechnology, scaffolds are now being developed that meet most of the requisites.

The technology of tissue engineering has evolved from the development of biological materials (biomaterials) and refers to the practice of combining scaffolds, cells, and biologically active molecules of functional tissues. The aim of tissue engineering is to gather functional structures that restore, maintain, or improve damaged tissues or full organs. Artificial engineered skin and cartilage tissues are examples that have been recently authorized by the FDA [2].

The operation is usually initiated by building a scaffold from a wide range of potential sources, from proteins to plastics. When scaffolds are created, cells with or without a "mix" of growth factors can be introduced. Assuming that the environment is appropriate, the tissue grows. Sometimes, cells, scaffolds, and growth factors are mixed together simultaneously, giving the tissue the opportunity to "self-assemble" [2].

Different ways to create a new fabric or tissue is using the present scaffold. The donor tissue or organ cells are stripped and the maintained collagen scaffold is used to form a new tissue. A new tissue has been created in the biological engineering of the heart, liver, lungs, and kidney tissues in rat. This approach holds great promise for the use of scaffolds from human tissues that are discarded during surgery and integrated with the patient's own cells for the work of dedicated members that cannot be rejected by the immune system.

The tissue needs a good "draining and plumbing system" (veins or arteries), a way to feed nutrients into cells and carry waste. Without blood supply or any similar mechanism, cells die quickly. Ideally, scientists would like to be able to create engineered tissue using a plumbing system that has already been built (lattices). New hope for the bum knee: cartilage has been very difficult, if it is not impossible to repair since cartilage lacks a blood supply to promote regeneration. The gel/adhesive combo was successful in regenerating cartilage tissue following surgery in a recent clinical trial of patients.

5. The main goal of tissue regeneration

The main goal of tissue regeneration studies is to acquire knowledge that will enhance the new wide range of regenerative medicine. This information may include evidence to stimulate stem cell activity, structural engineering of better scaffolds or direct initiation of biologic regeneration programs. Scientists already understand some forms of regeneration enough to manipulate and modify major events for therapeutic reasons. For example, the common practice of bone marrow transplantation is to properly guide hematopoietic cells to regenerated blood cells. However, for most examples of innovation, research has begun to acquire knowledge and techniques to try to ban or enhance selective steps selectively during renewal.

5.1. Musculoskeletal tissues

Musculoskeletal injuries impact millions of people globally and affect their health and well-being as well as of their companion and athletic animals. Soft-tissue injuries represent almost half of these and are associated with unorganized scar tissue formation and long time-depending healing processes. Cell based therapeutic strategies have been developed in the past decades aiming at the treatment and reversion of such disorders. Stem cells are appealing in the field, being a responsive undifferentiated population, with ability to self-renew and differentiate into different lineages. Mesenchymal stem cells can be obtained from several adult tissues, including the synovial membrane. Synovia-derived mesenchymal stem cells can be found in individuals of any age and are associated to intrinsic regenerative processes, through both paracrine and cell-to-cell interactions, thus, contributing to host healing capacity. Studies have demonstrated the potential benefit of synovia-derived mesenchymal stem cells in these regenerative processes in both human and veterinary medicine.

5.2. Bone regeneration

Bone regeneration is a surgical technique (**Figure 4**) that uses barrier membranes to direct, or guide, the growth of new bone at the site of the defect. The principle is that the barrier

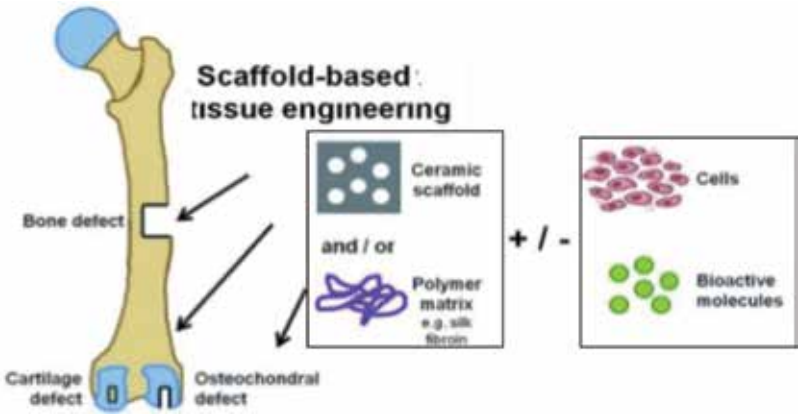

Figure 4. Bone scaffold: the bone capacity or the osteogenic potential of a bone graft is given by cells involved in bone formation, such as mesenchymal stem cells, osteoblasts, and osteocytes. The term osteoconductive refers to the scaffold or matrix which stimulates bone cells to grow on its surface [4].

membranes create and maintain a space above the bone defect; this allows the slower mesenchymal cells with osteogenic potential to populate the defect and regenerate without interference from the more quickly proliferating overlying soft tissues. Protection of the clot in the defect, exclusion of gingival connective tissue cells, and preparation of an enclosed space in which osteogenic cells can migrate from the bone are three essential elements of a successful outcome. Many types of grafts have been used as space maintainers between the membrane and the bone defect. Autografts, allografts, and xenografts have all been used successfully, either alone or in combination, for bone regeneration using particulate materials.

5.3. Applications

Tissue engineering currently plays a relatively small role in treating patients. Additional bladder, small arteries, skin grafts, cartilage, and even the entire trachea have been implanted in patients but the operation is still experimental and of high cost. While the more complex organ tissues such as heart, lung, and hepatic tissue have been successfully reconstituted in the laboratory, they are far from being entirely cloned and ready for transplantation in a patient. These tissues, however, can be very useful in research, especially in drug research [2].

Researchers have developed multi-capacity (pluripotent) stem cells that can be transformed into any type of cell in different types of specific areas and found that they controlled by very specific gene networks that determine the fate of cells. Most other medical research has focused on multivariate stem cells to modify the range of growth solutions in which cells are placed. Bone marrow stem cells in mature cells have been able to take stem cells along the way from multiple-capacity to bone maturation that can be implanted in a patient.

The ability to regenerate a new kidney from a patient's own cells would provide major relief for the hundreds of thousands of patients suffering from kidney disease. The resulting organ tissue was able to remove metabolites, re-absorb nutrients, and produce urine both in vitro and in vivo in rats. This process has been used previously in the heart, liver, and lung tissue.

The development of implantable tissue to replace renal function permanently is a promising hope in overcoming donor deficiency problems and morbidity associated with immunosuppression in transplantation.

One of the main challenges researchers face when trying to cultivate tissue engineering organs is to produce a scaffold, in which new cells can be implanted. While some scientists have followed three-dimensional printed scaffolds, many others focused on decellularization of local tissues to produce non-cellular scaffolds. The decellularization process typically consists of a series of perfused detergents through the organ, stripping the cells and nuclear material behind, and leaving the extracellular matrix. When developing decellularization protocols, researchers must balance the need for cellular material elimination with the need to maintain the properties of an extracellular matrix important.

Humans have limited regeneration ability, all the organ tissues can regrow, but it is very limited except the liver. Studies can find new methods to deal with regeneration. Recently, scientists are investigating the genes and factors which are active during regeneration. Scientists already understand some forms of regeneration sufficiently to manipulate and modify key events for therapeutic causes. So, in future, people will not need to use prosthesis, it will be more comfortable than using prosthesis because limbs will not lose their function and the regeneration of disabled people.

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Section 2

Bio-Nano-Materials for Tissue Regeneration

Chapter 2

Gelatin and Collagen Nanofiber Scaffolds for Tissue Engineering

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José Manuel Cornejo Bravo,
Irma Esthela Soria Mercado and
Luis Jesús Villarreal Gómez

Additional information is available at the end of the chapter

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Abstract

One of the main complications that can present a person with second and third degree burns is the possibility of being infected by opportunistic bacteria or viruses that are present in the environment. Nowadays, the majority of the burn injuries are treated with conventional gauze, which involves a high probability of infection and pain for the patient being treated with this method. In order to obtain low-cost scaffolds, natural and abundant polymers were used such as gelatin (GEL) and collagen (COL). The GEL functions as a base scaffold, stable and flexible, and also biocompatible because it is a byproduct of the partial hydrolysis of COL, which is an indispensable component for the stability of the cell membrane and it is present in great extent in the human epithelium.

Keywords: cutaneous dressings, polymer, gelatin, collagen, bioactivity

1. Introduction

Electrospinning technique is used for the production of fibers at nanometer scale, which has been used previously for the production of cutaneous dressings and a great variety of scaffolds with biomedical interest. It consists of the injection of a polymer solution properly homogenized in a polar solvent, in order to obtain a conductive material; by applying a current of the order of kilovolts (kV), it allows the solution to form a Taylor cone, which permits the formation of fibers. Another widely used technique is the electrospray that starts from the same principle of electrospinning but through a solution that allows the formation of suspended nanoparticles in the solvent, so that they are deposited on the collector (**Figure 1**) [1].

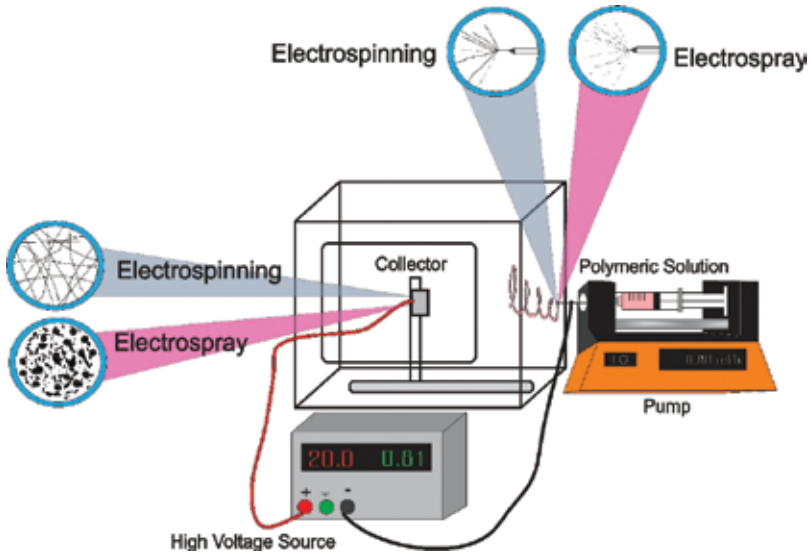

Figure 1. Electrospinning/electrospray scheme with polymer solutions.

Generally, the polymers used in electrospinning for biomedical applications are biodegradable and biocompatible; thus, they can be in contact with physiological medium without generating undesired reactions. Among them is GEL, which is a natural polymer obtained from collagen (COL) which is a protein obtained from the connective tissue of animals when boiled in water, GEL is a very useful polymer in electrospinning because of its ability to produce fibers of nanometric scale independently of the changes in the temperature and humidity of the environment, for this reason it was used as a base for the formation of bioactive electrospun [2].

Electrospinning has recently been extensively studied; it is a well-known technique for the manufacture of nanoscale fibers because of its various advantages such as high surface-volume ratio, adjustable porosity, and ease of surface functionalization. The resulting fibers are extremely useful for applications in the fields of tissue engineering, drug delivery, and wound dressing. In addition to the morphological, physical, and chemical properties, electrospun scaffolds are often evaluated through various cell studies. Researchers have adopted approaches such as surface modification and drug loading to improve scaffold ownership and function [3].

The electrospinning technique has been used as an efficient and accessible method for the manufacture of nanofibers with a wide variety of applications in the fields of pharmaceutics and medicine. Among the most outstanding applications, we can see wound dressings, drug delivery systems, or tissue engineering scaffolds [4]. Animal polysaccharides such as chitosan, hyaluronic acid, heparin, and collagen have been studied with this technique; these compounds are natural biopolymers with numerous advantages for biomedical applications such as biocompatibility, biodegradability, nonantigenicity, and nontoxicity [5].

2. Gelatin electrospun scaffolds

GEL nanofibers have been prepared using an electrospinning process in previous studies. To improve water-resistant capacity and thermomechanical performance for potential biomedical applications, GEL nanofibers were cross-linked with glutaraldehyde-saturated steam at room temperature. Exposure of this nanofibrous material to the glutaraldehyde vapor was performed for 3 days to provide sufficient cross-linking to preserve the fibrous morphology assayed by immersion at 37°C warm water. On the other hand, cross-linking also led to improved thermostability and substantial improvement in mechanical properties. The denaturation temperature corresponding to the transition from the helix to the coiled structure of the air-dried samples increased by about 11°C and the tensile strength and modulus were nearly 10 times greater than those of the electrospun GEL fibers. In addition, cytotoxicity was evaluated based on a cell proliferation study by culturing human dermal fibroblasts on the fibrous scaffolds of cross-linked GEL for 1, 3, 5, and 7 days. It was found that cell growth occurred and increased almost linearly over the course of the entire cell culture period. Initial inhibition of cell growth on the cross-linked fibrous substrate of GEL suggested some cytotoxic effect of residual glutaraldehyde on cells [6].

The GEL was successfully electrospun using a solvent based on ethyl acetate, acetic acid, and water. Since natural polymers including GEL have limited solubility in water, toxic or highly acidic solvents are usually used to dissolve them for electrospinning. Instead of using such solvents, ethyl acetate was used with acetic acid in water; the beneficial effect of its use was investigated in terms of the spinning capacity of the nanofiber and the acidity of the solvent. It was found that the substitution of acetic acid with ethyl acetate improved the spinning capacity of the nanofiber by reducing the surface tension of the solution, as well as increasing the pH of the solvent significantly. The optimum composition of the co-solvent was found to correspond to a ratio of ethyl acetate to acetic acid in a ratio of 2:3. Under this solvent condition, the GEL could be dissolved at concentrations up to 11% by weight and successfully electrospun to produce nanofibers of various diameters (47–145 nm on average) depending on the GEL concentration. The water-based co-solvent method proposed herein may be useful for generating other natural nanofibrous polymers, as well as being applicable in delivery systems for bioactive molecules within the nanofiber matrices [7].

In another study, electrospinning was performed in aqueous GEL solution, increasing the spinning temperature. To improve stability and mechanical properties in the wet state, the GEL nanofibrous membrane is chemically reticulated by 1-ethyl-3-dimethylaminopropylcarbodiimide hydrochloride and N-hydroxyl succinimide. The crosslinker concentration was optimized by measuring the degree of swelling and weight loss. The nanofibrous structure of the membrane was maintained after lyophilization, although the fibers were crimped and conglutinated. The tensile test revealed that the hydrated membrane becomes flexible and provides predetermined mechanical properties [8].

It can be said that electrospinning has been one of the simplest, most versatile, and promising processes for producing continuous nanofibers. In the case of GEL, this polymer has been

widely used in food for the purpose of thickening and stabilization. At nanometer scale, electrospun nickel/GEL nanofibers can be used in food for the same purpose in smaller quantities that give more results that are efficient. A study investigated the influence of the parameters that affected during electrospinning on the properties of the electrospun GEL. GEL concentrations of 7 and 20% (w/v) were electrospun under 28 or 35 kV applied voltage. The feed rate was 1 or 0.1 ml/h. Before electrospinning, the electrical conductivity, surface tension, and rheological properties of the feed solutions were determined. Morphological analysis showed that only the 20% GEL solution produced nanofibers. The electrical conductivity, surface tension, consistency index, and flowability of the 20% GEL solution were 4.77 mS/cm, 34.91 mN/m, 1.37 Pa, and 0.93 sn, respectively. The range of diameters of nanofibers increased with the applied voltage. The zeta potential and diffusion coefficients of dispersions containing the GEL and the electrospun GEL were determined. Both values were higher for dispersions containing electrospun GEL than for dispersions with GEL at the same concentration. The zeta potential and diffusion coefficient values of dispersion coefficients containing electrospun GEL decreased as the voltage applied during the electrospinning increased. The applied low voltage resulted in higher values of the diffusion coefficient and zeta potentials for dispersions containing electrospun GEL nanofibers, which may indicate that these nanofibers can be used for the stabilization of food emulsions, as compared to the smooth nanofiber morphology without pearl formation obtained at the highest stress [9].

On the other hand, electrospinning is a very useful technique for producing polymeric nanofibers by the application of electrostatic forces. It has been reported that the modeling and optimization of the GEL/chitosan electrospinning process have been achieved, where the interaction effects of the GEL/chitosan blend ratio (50/50, 60/40, and 70/30) were investigated, the applied voltage (20, 25, and 30 kV), and the feed rate (0.2, 0.4, and 0.6 ml /h) on the optical fiber diameter and the standard deviation of the fiber diameter by scanning electron microscopy. To manufacture the GEL/chitosan nanofibers mixture, trifluoroacetic acid/dichloromethane was selected as the solvent system. The model obtained for the hinge diameter has a quadratic relation with the applied voltage and the feed rate. The interaction between the applied voltage and the flow rate was found to be significant, but the interactions of the mixing ratio and the flow and the mixing ratio and the applied voltage were insignificant. Scanning electron micrographs of human dermal fibroblast cells in the structure of gel/chitosan nanofibers show coupling and proliferation on the surface of fabricated scaffolds. These microfibers have great potential to be used as scaffolding for the engineering of cutaneous tissues [10].

In another study, poly (lactic-co-glycolic acid) (PLGA) and PLGA/GEL nanofibrous sheet materials embedded with mesoporous silica nanoparticles were fabricated using an electrospinning method. The mean diameters of the nanofibers were 641 ± 24 nm for pure PLGA scaffolds versus 418 ± 85 and 267 ± 58 nm for PLGA scaffolds/silica nanoparticles at 10% w/w PLGA/GEL silica nanoparticles to 10% w/w, respectively. The results of the contact angle measurement (102°± 6.7 for the pure PLGA scaffold versus 81 ± 6.8 and 18 ± 8.7 for PLGA/silica nanoparticles and the PLGA/GEL scaffolds/silica nanoparticles revealed improved hydrophobicity of scaffolds by the incorporation of GEL and silica nanoparticles. In addition, the incorporation of scaffolds with silica nanoparticles resulted in better tensile mechanical properties. The culture of PC12 cells in the scaffolds showed the

introduction of silica nanoparticles into the matrices. The DAPI staining results indicated that cell proliferations in the PLGA/silica and PLGA/GEL/silica nanoparticle matrices were surprising (almost 2.5%), and the proliferation of PLGA and PLGA/GEL leads to improved cell attachment and proliferation (three times, respectively) higher than in pure aligned PLGA scaffolds. These results suggest superior properties of mesoporous silica nanoparticles incorporated into electrospun PLGA/GEL scaffolds for stem cell culture and tissue engineering applications [11].

On the other hand, we studied the mass production of GEL nanofibers by spiral electrospinning and investigated the performance of different cross-linking methods such as glutaraldehyde vapor and liquid phase cross-linking. Compared with conventional single-needle electrospinning, spiral electrospinning nanofibers were finer, where an increase of more than 1000 times over the productivity of traditionally obtained nanofibers was obtained. Mechanical tests showed that the tensile strength of nanofiber membranes increased from 1.33 to 2.60 MPa after cross-linking of glutaraldehyde vapor and from 1.33 to 5.08 MPa after liquid phase cross-linking. In addition, the SEM and FTIR analysis indicated that the membrane of nanofibers obtained by liquid phase cross-linking presented better properties, resulting in an ideal material for wound dressing applications [12].

A study where GEL scaffolds were prepared by electrospinning using aqueous acetic acid and thermally cross-linked with glucose showed the effect of the amount of glucose used as the cross-linking agent on the mechanical properties of the GEL fibers, and it was found that cross-linking with glucose increases the elastic modulus of GEL fibers from 0.3 GPa to 0% glucose content at 1.1 GPa with 15% glucose. This makes fibrous GEL scaffolds cross-linked by glucose, a promising material for biomedical applications [13].

In another study, GEL nanofibers were prepared from ternary mixtures of GEL/acetic acid/water with the aim of studying the feasibility of making GEL nanofiber membranes at room temperature using an alternative benign solvent by significantly reducing the concentration of acetic acid. The results showed that GEL nanofibers can be optimally electrospun with a low concentration of acetic acid (25%, v/v) combined with GEL concentrations above 300 mg/ml. GEL mats made from low acetic acid solutions had some advantages such as maintaining the decomposition temperature of the pure GEL (230°C) and the reduction of the acid content in the electrospun mats, which allowed a cellular viability of more than 90% [14].

The manufacture of fibroin nanofibers/silica GEL loaded with ceftazidime without the loss of structure and bioactivity of the drug was demonstrated using the electrospinning method. The results show that the average diameter of the drug-loaded nanofibers at the optimum ratio of polymer to drug [10, 1] was 276.55 ± 35.8 nm, while increasing the feed ratio to 1:1 increased the mean diameter at 825.02 ± 70.3 nm. In FTIR analysis of drug loaded on the nanofibers revealed that the drug ceftazidime was successfully encapsulated in the nanofibers while the feasibility study approved the cytocompatibility of the system. The drug was released from the nanofibers for 6 h, and the formation of the zone of inhibition in the diffusion test demonstrated the antibacterial effect of the drug-loaded nanofibers. Together, the fibroin/GEL/ceftazidime nanofibrous system can enhance drug effectiveness particularly in the prevention of postsurgical adhesions and wound dressing infections [15].

Since GEL undergoes a gelation process, Furuike et al., used a new dry spinning process for GEL. In this case, the nonwoven GEL fabrics were electrospun by applying dry spinning principles. The diameter of the fibers, the viscosity, and flow rate of the solution depended directly on the GEL concentration. Spunted nonwoven fabrics with a concentration of 25% (w/w) GEL exhibited a nanoscale diameter. In order to improve the properties of the nonwoven fabrics, they were cross-linked with glutaraldehyde vapor after spinning by the addition of N-acetyl-d-glucosamine to the GEL solution before spinning followed by heating of these fibers. Nonwoven fabrics cross-linked by glutaraldehyde vapor exhibited improved mechanical properties compared to those without cross-linking or cross-linking of N-acetyl-d-glucosamine. Swelling and water absorption did not produce morphological changes in glutaraldehyde cross-linked fibers. The thermogravimetric analysis (TGA) thermogram did not confirm any phase change in the composite structure. In addition, in vitro cytocompatibility studies using human mesenchymal stem cells showed the compatible nature of the developed nonwoven fibers, where they demonstrated that these nonwoven fibers could be useful in medical care [16].

Delivery of hydrophobic drug into the hydrophilic polymer matrix as a carrier is usually a challenge. Therefore, in one study, the synthesis of GEL nanofibers by electrospinning was presented, which were evaluated as a potential carrier for the oral system of hydrophobic drugs, piperine. GEL nanofibers were cross-linked by exposing to saturated glutaraldehyde vapor, to improve their water-resistive properties. An exposure of only 6 min was not only adequate to control early degradation with intact fiber morphology, but also significantly marginalized any adverse effects associated with the use of glutaraldehyde. The results illustrated good compatibility of the hydrophobic drug in GEL nanofibers with promising patterns of controlled drug release by varying the cross-linking time and the pH of the release medium [17].

3. Collagen electrospinning

Collagen (COL) is the most widely distributed class of proteins in the human body. The use of COL-based biomaterials in the field of tissue engineering applications has been growing strongly during the last decades. It is for this reason that multiple cross-linking methods have been investigated and different combinations with other biopolymers have been explored to improve tissue function. The COL has a great advantage, as it is biodegradable, biocompatible, readily available, and highly versatile. However, since COL is a protein, it remains difficult to sterilize without alterations in its structure [18].

We have investigated the possibility of preparing COL-inspired nanofibers by electrospinning aqueous suspension of telopeptide-free COL molecules avoiding organic solvents and blends with synthetic and natural polymers. The results underscored the need for a basic atmosphere between the needle and the ground collector in order to increase the pH of the environment during auto-assembly of COL molecules along the electrostatic force lines in order to prepare a biomimetic component of reinforcement of new biomaterials for medical and surgical use [19].

It has been reported that he designed a durable sandwich wrap preparation system with high liquid absorption, biocompatible, and with antibacterial properties. For this purpose, various weight ratios of the COL solution to chitosan were used to immobilize on the polypropylene

nonwoven fabric, which was pre-grafted with acrylic acid or N-isopropyl acrylamide to construct a durable wound sandwich liner membrane with high water absorption, easy removal, and antibacterial activity in an animal skin model. The results indicated that tissue immobilized with N-isopropyl acrylamide and COL/chitosan/PP/N-isopropyl acrylamide-COL/chitosan) showed a better healing effect than COL/chitosan immobilized polypropylene tissue. The poly (propylene)/N-isopropyl acrylamide/COL/chitosan-treated wound showed an excellent remodeling effect on histological examination with respect to the construction of the vein, epidermis, and dermis at 21 days post-cutaneous lesion. The water absorption values and water diffusion coefficient for polypropylene/N-isopropyl acrylamide/COL/chitosan were higher than those of polypropylene /acrylic acid/COL/chitosan under a weight-volume ratio of COL/chitosan. Both polypropylene/N-isopropyl acrylamide/COL/chitosan and poly (propylene)/acrylic acid/COL/chitosan showed antibacterial activity [20].

Electrospinning is a process that is used to create nanofibers, which have the potential to be used in many medical and industrial applications. The molecular structure of the raw material is an important factor in determining the structure and quality of the electro-chip fibers. COL has been extracted from a cold-water hoki species (*Macruronus novaezelandiae*), and this was prepared in several different molecular formats (triple helical CO, denatured whole chains, denatured atelocollagen chains, and GEL) for electrospinning. When denatured COL chains were used, 10% acetic acid proved to be an aqueous solvent effective to produce uniform fibers. This information is useful for the development of a nontoxic aqueous solvent system suitable for the industrial enlargement of the electro-silting process [21].

Nerve tissue engineering is one of the most promising methods in nerve tissue regeneration. The development of combined scaffolds of COL and glycosaminoglycans can potentially be used in many soft tissue-engineering applications. In a study by Timnak *et al*. developed two types of randomized and aligned electro-alloying. Their cellular tests showed that the scaffold acted as a positive factor to support the growth of connective tissue cells. These results suggested that scaffolding of nanostructured porous COL-glycosaminoglycans is a potential cell carrier in nerve tissue engineering [22].

On the other hand, COL and hyaluronic acid are the main components of the extracellular matrix naturally and have been successfully used in the electrospinning. In this case, a solution of COL/hyaluronic acid polymer was electrospun creating a scaffold for patients with osteoporosis who have reduced bone strength. The membranes were cross-linked to render them insoluble and conjugated to gold nanoparticles to promote biocompatibility. Their results showed that COL/hyaluronic acid scaffolds were insoluble in aqueous solutions and promoted cell fixation that could be used as a tissue engineering framework to promote cell growth [23].

Zulkifli *et al*. [24] focused on the degradation behavior of nanofibrous scaffolds composed of HEC/PVAL (alcohol hydroxyethyl cellulose/polyvinyl alcohol) and HEC/PVAL/COL as potential substrates for the engineering of cutaneous tissues in two media (PBS) and Dulbecco's modified Eagle's medium (DMEM) for a period of 12 weeks of incubation. Once the scaffolds were characterized, the HEC/PVAL/COL scaffolds showed a slower degradation rate in both media compared to the HEC/PVAL blend nanofibers. All fibers showed irregular and rough surfaces toward the final week of incubation in PBS and DMEM solution. As the degradation time increased, there were few changes in the chemical structure determined

by the FTIR spectra, while the thermal studies revealed that the melting and crystallinity temperatures of the scaffolds were slightly shifted to a lower value. Both HEC/PVAL and HEC/PVAL/COL fibers showed a significant decrease in Young's modulus and tensile stress during the 12 weeks of degradation. Their results demonstrate that these nanofibrous scaffolds showed degradation behavior that meets the requirements as a degradable biomaterial potential for dermal replacement.

The development of biomaterials with the capacity to induce the healing of cutaneous wounds is a great challenge in biomedicine. In one study, COL sponges were developed from tilapia skin and electro-nylon nanofibers for wound dressing. It was found that nanofibers could significantly promote the proliferation of human keratinocytes and stimulate epidermal differentiation through the expression of regulated genes involved, filaggrin and type I transglutaminase in human keratinocytes. In addition, COL nanofibers could also facilitate the regeneration of rat skin, in one study, electrolyzed nanofibers of COL were prepared from biomimetic tilapia skin and were shown to have a good bioactivity and could accelerate the healing of wounds from rat quickly and efficiently. These biological effects can be attributed to the structure of the biomimetic extracellular matrix and to the multiple amino acids of the COL nanofibers. Therefore, tilapia COL nanofibers could be used as a new wound dressing, effectively avoiding the risk of transmitting diseases in future clinical application [25].

Another study using the double-extrusion electrospinning technique prepared with multilayer 3D scaffolds stacking poly-lactic-co-glycolic acid (PLGA) microfiber membranes alternately to micro- /nano-mixed fibrous membranes of PLGA and COL. The density of the COL fibers in multilayered scaffolds obtained was able to control the adhesion, proliferation, and osteogenic differentiation of MC3T3-E1 cells. Demonstrating that the homogeneous dispersion of glutamic acid-modified hydroxyapatite nanoparticles (nHA-GA) in the COL solution improved the osteogenic properties of the multilayer scaffolds fabricated. In addition, it found that PLGA-COL-HA micro-nano fibrous scaffolds were highly bioactive compared to pristine microfibrous PLGA and PLGA and COL micro/nano-mixed fibrous platforms [26].

The development of biomimetic scaffolds represents a promising direction in the engineering of bone tissue. This was demonstrated by Ma *et al.* [27], when they designed a two-step process to prepare a type of biomimetic hybrid hydrogels that were composed of COL, hydroxyapatite, and alendronate, the latter as anti-osteoporosis drug. These hybrid hydrogels of collagen, hydroxyapatite, and alendronate exhibited remarkably improved mechanical properties (G: 38–187 kPa storage modulus), higher gel contents, and lower swelling proportions compared to hydrogels prepared from COL only under similar conditions. In addition, they showed degradable behaviors against collagenase. The hybrid hydrogels of COL-hydroxyapatite-alendronate well supported the adhesion and growth of MC3T3-E1 osteoblastic cells. Such resistant but enzymatically degradable hybrid hydrogels hold the potential as scaffolds for bone tissue engineering.

The hybrid constructs from marine organism material for porous scaffolds of COL, such as fibrillated jellyfish and alginate hydrogel, mimic the two major components of cartilage,

thus being a promising approach as a model for the chondrogenic differentiation of mesenchymal stem cells human beings. This is why Pustlauk *et al.* [28] investigated their potential for joint cartilage repair. They studied the expression of the COL 2 gene and found that its expression was comparable in all scaffold types examined. However, the COL 2/COL 1 ratio was higher for pure alginate disks and alginate-cell suspension scaffolds compared to alginate-embedded stem cells. In addition, they found that the secretion of sulfated glycosaminoglycans was comparable in the suspension of alginate cells and cells embedded in alginate scaffolds. They conclude that hybrid COL constructs of jellyfish and alginate support the chondrogenic differentiation of stem cells and provide more stable constructs compared to pure hydrogels.

4. Electrospinning with the GEL/COL system

Angarano *et al.* [29] synthesized GEL and COL cross-linked fibers by the reactive electrospinning technique using a mixture of nontoxic solvents: acetic acid, ethyl acetate, and water (5, 3, and 2 w/w/w), eliminating fluorinated solvents, which require post-treatment and purification by the implementation of glyoxal, represented an easy, versatile, and one-step procedure. Enabling the expansion and fabrication of synthetic fabrics of COL based on nanofiber cross-linked GEL in situ. This in situ cross-linking renders the water soluble GEL fibers water resistant without adversely affecting the hydrophilicity, excellent wetting of fibers, cell compatibility, reabsorption, cell adhesion, and proliferation typical of COL nonwoven nanofibers cross-linked.

Tylingo *et al*. [30] prepared and characterized new porous scaffolds composed of chitosan, COL, and GEL for the preparation of GEL and COL scaffolds isolated from fish skin with various physicochemical properties. All biomaterials obtained showed homogeneous porosity. The type of protein polymer determined the rheology and mechanical properties of the preparation of the preparations. The use of protein polymers decreased the swelling ability of the materials by about 30% compared to the materials obtained from chitosan. GEL-containing materials showed the highest solubility (approximately 30%). Scaffolds obtained in 100% chitosan were found to be harder than COL materials by an average of 30% and less flexible more than twice. In addition, materials containing protein polymers showed good antioxidant properties.

In **Table 1**, other studies with the electrospinning technique are summarized.

Polymers	Application	Characteristics	References
GEL (type B porcine)	Food industry	Soft nanofibers without pearl formation	[9]
GEL (type B porcine)	Tissue engineering Cell regeneration in fibroblasts (BJ-5ta) and human embryonic kidney cells (HEK 293 T)	Nanofibers with up to 90% cell proliferation	[14]
GEL (type A porcine)	Tissue engineering Cell regeneration in fibroblasts (3 T3)	Nanofibers cross-linked in glutaraldehyde.	[5]

Polymers	Application	Characteristics	References
GEL (25.43 kDa)	Regeneration of Periodontal Tissues.	Reticulated membrane, hydrated and flexible.	[8]
GEL (bovine Type B)	Delivery system for bioactive molecules in nanofiber matrices	Nanofibers between 47 and 147 nm.	[7]
GEL	Scaffolds for cell regeneration in human gingival keratinocytes and human gingival fibroblasts.	Water-resistant glyoxal cross-linked nanofibers.	[29]
GEL/fibroin	Tissue engineering Cell regeneration in human fibroblasts	Nanofibers loaded with ceftazidime as an antimicrobial agent.	[15]
COL (type I)/poly (lactic-co-glycolic acid)/hydroxyapatite	Tissue engineering Osteogenic differentiation of MC3T3-E1 cells	Micro/nano-bioactive scaffolds	[26]
COL/hyaluronic acid	Tissue Engineering Regeneration of bone tissues in murine fibroblasts (L - 929)	Fibers insoluble in aqueous solutions that promote cell binding.	[23]
COL Free telopeptide type I (equine tendon)	Biomedical and Surgical	Simple molecular set of nanometric size	[19]

Table 1. Gelatin and collagen uses in biomedical applications.

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Chapter 3

Trends in Tissue Regeneration: Bio-Nanomaterials

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Abstract

Tissue engineering requires functional platforms or scaffolds with specific properties concerning the morphology, chemistry of the surface and interconnectivity to promote cell adhesion and proliferation. These requisites are not only important for cellular migration but also to supply nutrients and expulsion of waste molecules. Cell type must be considered when designing a specific cellular grown system as a scaffold; for instance, if they are autologous, allogeneic or xenogeneic. The challenge in tissue engineering is to develop an organized three-dimensional architecture with functional characteristics that mimic the extracellular matrix. In this regard, with the advent of nanotechnology scaffolds are now being developed that meet most of the aforementioned requisites. In the present chapter, the use of biopolymers based nanostructures is addressed, including biomaterials and stem cells, bio-nanocomposites, and specific clinical cases where these systems were employed. We emphasize the future challenges and perspectives in the design of biocompatible and nontoxic nanocomposites with high efficiency as a promoter for tissue regeneration and many other biomedical applications.

Keywords: antimicrobial, nanomaterials, nanoparticles, regenerative medicine, tissue engineering

1. Introduction

As part of the novel scientific challenges, the use and design of biocompatible and functional materials based on nanostructured systems have gained industrial and scientific interest due

to the surface of the material shows an exponential increase in their contact area, which can enhance significantly the physicochemical properties. In this regard, nano-sized particles (ranging from 1 to 100 nm) have been considered as an effective strategy for pharmaceutical carriers, antibacterial and skin regenerator systems [1, 2].

Nanotechnology research has been intensively developed over the last decades; it is rapidly expanding and providing significant contributions to materials science. The main reasons for its success are the interesting properties of nanostructures that have led to greater efficacy systems, based on their physical dimension, shape, and composition [3]. Nowadays, these materials represent a broad potential for market growth, and recently these are commercialized as nanotechnology-enhanced products [4]. In the majority of these products, the presence of nanoparticles (NPs) is related to the addition of reinforcing agents such as additives, to improve physical/chemical or antibacterial characteristics.

The overall standings of NPs as additives involve organic systems, such as polymers [5], lipids [6], dendrimers [7], nano gels [8], nano emulsions [9], supramolecular structures [10] and others [11, 12]. In particular, inorganic NPs used for tissue regeneration such as carbon nanostructures (graphene, carbon nanotubes, fullerenes) [13], metallic nanoparticles, (such as silver, copper gold, titanium dioxide), quantum dots [14], and magnetic nanoparticles [15] have also been described.

Recent advances in the use of nano-sized particles in pharmaceutics involves the design of controlled drug delivery systems [16], biomarkers as diseases detection [2], pathogen/protein identification [17], molecule separation/purification [18] and regenerative medicine approaches [17, 19, 20]. Recent studies have been focused in biomedicine in order to execute multidisciplinary research, combining topics such as chemistry, biology, physics, engineering and materials science; associated with the design of functional systems, addressed to the tissue regeneration responses in organisms. The development of tissue engineered systems from health sciences is aimed to promote specific cell growth to replace tissue damage, associated with diseases such as cancer, trauma, hepatitis or congenital malformations [21].

In this chapter, the recent trends in the use of nanostructured systems combined with biopolymers will be discussed, divided into three parts: biomaterials and STEM cells, bio-nanocomposites and the current clinical cases where these systems were employed; aiming to emphasize the future challenge and perspectives in the design of biocompatible and nontoxic nanocomposites with high efficiency as promoter for tissue regeneration and many other biomedical applications.

2. Biomaterials and stem cells

Tissues in the human organism are generated, maintained and repopulated by stem cells. These are specialized cells capable of cell renewal and are able to differentiate into the different cell types in the human body. Stem cells have several differentiation programs, therefore, they possess information to allow them to become any cell in the body or a restricted cell type with a specialized function. These abilities make stem cells extremely useful for biomedical applications and regenerative medicine and have become the main molecular tool for these purposes [22].

Stem cells are derived from three primary sources, the embryonic origin, the mesenchymal origin and the so-called induced pluripotent stem cells. Cells from the embryonic origin are obtained from the inner cell mass of the blastocyst. They are considered a very important cell source for cell replacement therapies and have been used in regenerative medicine approaches by virtue of their ability to differentiate into any adult cell type [18]. Ethical considerations have restricted their use in many countries.

Cells from the mesenchymal origin, as opposed to embryonic stem cells, come from adult organisms, and these cells can differentiate into cell lineages organ-specific, the use of these cells in regenerative medicine makes them very appropriated because the lack of ethical concerns of obtaining cells from embryos. As in other tissues, cartilage self-renewal potential is limited due to the absence of a dense population of progenitor cells, multipotent mesenchymal stem cells, have been used therapeutically for the purposes of cartilage repair. Arthrosis of the carpometacarpal joint is common in postmenopausal women and requires surgical treatment; mesenchymal stem cells have been therapeutically used as connective tissue progenitor donors isolated from the anterior and posterior iliac crest. Treatment with mesenchymal stem cells is a very effective therapeutic alternative, the patient avoids surgery and greatly improves articular function and diminishes pain [23].

The so-called induced pluripotent stem cells are adult cells with a modified genetic program, which have gained potency due to transcription factor transfection. According to Mall and Wernig [24], cell reprogramming makes now possible to change cell fate and transform adult skin cells into neurons, hepatocytes or cardiac cells. This approach is useful for many biomedical applications from studying disease progression as well as the efficacy and safety of newly developed drugs even before animal testing on clinical trials [24].

Stem cells have been successfully used to develop organoids. Organoids are stem cell 3D cultures resembling real organs. Cells in this array have interactions with each other, as well as with the extracellular matrix, which are not seen in petri dish monolayer culture [25]. An example in the advance of organoids came from the idea to perform functional studies in human brains, which is not that easy to address due to difficulties to perform studies in whole human brains or the inaccuracies of using postmortem tissue, therefore, researchers were in need of an *in vitro* model system that would mimic the characteristics of the brain during development. Three-dimensional *in vitro* brain models are arising, and more importantly, how they are now used to study the evolution of the brain and the associated neural disorders [26].

Stem cells have clinical potential for injury treatments and degenerative diseases. The challenge of the use of nanomaterials in these systems is related to the optimal control of microenvironment conditions to transplant cells [27]. The combined use of stem cells and nanoparticles has improved cell proliferation and differentiation, used in different diseases, such as ischemic stroke, spinal cord, multiple sclerosis, Parkinson, Alzheimer, and others [28].

In order to recapitulate the function and structure of the native extracellular matrix (ECM) to generate functional tissue, researchers have developed new biodegradable and biocompatible synthetic of natural polymer structures called scaffolds [29]. The supporting scaffold temporarily replaces the function of the ECM, supporting the 3D geometry and providing the appropriated structural conformation, enabling cell adherence, and facilitating the conformation of

a tissue with its functional properties [30]. The microscopic structure must allow nutrient diffusion as well as the efflux of metabolites no longer needed to the cell through the scaffold. Finally, the scaffold must have good mechanical properties, enabling handling during culture in bioreactors and transplantation into the host [30].

One of the biggest health issues worldwide is organ failure derived from disease or a traumatic event; this has been resolved by transplantation of organs from living or deceased patient donors. The list of donors and recipients has increased in the last years and there are many patients on waiting lists for organ donation [31]. According to Gilpin and Yang [31], tissue engineering consists of three important aspects: the participating cells, the signaling molecules used and the scaffold. Scaffolds can be natural or synthetic. Natural scaffolds are derived from decellularization processes using chemical, enzymatic or physical methods. The resulting decellularized scaffold has to be recellularized either with one or different type of cells, in other cases induced pluripotent stem cells are used to recapitulate organ functionality [31–33].

For more than 20 years, scientists started developing nano-bio-materials and it is thought that nano bio-composites will be more important than non-nanometric materials at the physiological level. The advancement in biomedical research due to the incorporation in biomaterials to biological models has had a great impact in health sciences [34].

Decellularized scaffolds have been improved by combining them with biomaterials, not only to provide the extracellular matrix required for the cells to proliferate and differentiate but also to provide structural, biochemical and biomechanical support to the regenerated organ. Cheng et al. [35], developed silk-based scaffolds for bone regeneration, but their therapeutic efficacy was not optimal, therefore they developed a composite material of mesoporous bioactive glass/silk scaffold to improve mesenchymal stem cell regeneration activity in a rodent model for postmenopausal osteoporosis. They proved that the composite material provided the optimal environment for mesenchymal stem cell differentiation, attachment, and proliferation as treatment of osteoporotic defects [35]. Sterling and Guelcher [36] proposed another example of scaffolds to heal fractures derive for osteoporosis. In this research, the authors have argued that bone autografts (bone sample from the same patient), that have been used to improve fractured bone healing, have some pitfalls due to the limited amounts of bone that can be harvested, instead, hybrid scaffolds have been fabricated made with silk and calcium phosphate to stimulate bone formation and to reverse bone loss. The same group has shown that local delivery of recombinant bone morphogenic protein from microspheres made with polylactic glycolic acid has improved the mechanical properties of vertebrae in animal models [36].

We have been addressing some examples of the use of nanomaterials in conjunction with biological models or cells, but we are also going to show how these systems have to be visualized for further biomedical characterization.

Regarding tissue engineering, once the bio-engineered tissue is developed, it has to be evaluated in its structure and function. Histological and histochemical techniques have been used. For example, it is important not only to evaluate the 3D structure of scaffolds and its possible interaction with cells prior deciding on a biological or clinical application, but also the functionality of the cells contained the manufactured tissues. Different imaging techniques can be used to assure the efficiency of the biocomposites, such as ultrasound, microscopy, magnetic resonance imaging (MRI), and other optical imaging techniques [20].

Confocal imaging is a very useful to imaging technique in biomedical research, offering the ability to visualize different cell structures and their interaction with nanomaterials by using fluorescent dyes, as well allowing the creation of Z-stacks to recreate the three-dimensional architecture. Confocal imaging has been used to analyze the safety of dental nanostructured materials made from methacrylate monomers [37]. Another approach of engineered tissues has been the generation of oral soft and non-soft tissue. Recent advances involve the regeneration of whole teeth. Cells dissociated from epithelium and mesenchymal tissue of tooth buds were used to create a bioengineered tooth in vitro: cells were seeded to biodegradable polyglycolic/polylactide scaffolds having the shape of a tooth and implanted to rat hosts for 30 weeks and tooth structures were obtained [38].

The use of cell combined with nanostructured materials has greatly improved translational research making now areas like biomedical research and nanomedicine, important contributors of many peer-reviewed papers, publications and funding in these areas have had an exponential increase since 2011. It is expected even a more dramatic increase in the years to come [39]. In accordance with these new developments, another branch of research has been developed, *nanotoxicology*. This increase in published data now has to be proved innocuous to the biological system or organism where is going to be applied. Eventually, this will lead to more research to discover the advantages or disadvantages of using nanostructured materials with potential biomedical applications. Toxicity of nanomaterials has to be verified at different levels, whereas is about the systemic effects or the inflammatory and immunological response toward them, as well as the intra or extracellular effects [39].

3. Bio-nanocomposites

Biomaterials research has been concerned with the use of nanomaterials to enhance the tissue regeneration process. In this regard, nanomaterials can be classified into organic and inorganic systems. Diversity in organic materials derived mainly from polymers, such as polysaccharides, collagen, and chitosan have been recently used with different morphologies into the biomedical application and stem cell differentiation [19]. In particular, the use of polymer NPs as carriers or drug delivery systems is promising materials used as neuroprotectors to avoid acute ischemic stroke, which is actually considered one of the most common causes of death worldwide [40]. Nanostructured drug delivery systems offer many advantages, such as the avoidance of drug degradation, the possibility to improve the pharmacokinetic profile and the specificity at nano scale.

NPs from different materials have been functionalized with bioactive molecules in order to describe their effects in cells and tissues. Bio-composites of silica NPs with fluorescent compounds from the tree *Eysenhardtia polystachya* were internalized into MCF-7 breast cancer cells and observed with confocal microscopy to analyze their possible anti-tumor effect [41].

Cells interact with each other through their own synthesized ECM, which provides support and allows proliferation and differentiation processes. In consequence, ECM produces high membrane adherence with specific ligands associated with signaling pathways and possible migration, which can regulate the cell growth [42, 43]. Our body possesses natural ECM, mainly

conformed by fibrous proteins and proteoglycans, ranging in size from 50 to 500 nm [44]. In this regard, collagen is an important source of ECM, present in the majority of connective tissues, such as bone, skin, and tendons. It is confirmed by a three-dimensional protein network by nano-sized fibers, with high resistance and adherence [29, 45], and recently, many studies have been focused to mimic this behavior and replace it with functional materials.

The challenge in the research of materials able to replace the ECM is the recreation of a functional nanostructured network which allows cellular growth and differentiation. In fact, there are a lot of techniques for this task but there is one in particular that has been used more frequently in the recent years by researchers because it actually generates a fibrous structure like the ECM [46]. Electro spinning technique can produce nano-sized fibers from different sources, such as polymers, biocompatible systems, sol-gel, and nanocomposite materials. This technique generates three-dimensional porous fibers with high electrostatic attraction, associated with their high surface area/aspect ratio [47]. In this regard, this technique works from a solution (polymer, nanocomposite, and others) passed through a syringe, ending from a Taylor cone to control the efflux. A voltage source creates a drop and is collected at different distances to create variable morphology fibers. The surface tension produced between the collector and the needle is created by the electrostatic forces of the fibers [47].

Chitosan (CTS) has been defined as one of the most common biopolymers and chemically is a linear polymer derived from the deacetylated process of chitin, which is obtained from crustaceans [48]. The main characteristics of CTS are their biocompatibility and degradability [49], and can be easily processed in many different structures such as films, scaffolds, and fibers. CTS has been studied as antibacterial, biocompatible material, as a carrier for specific drug delivery and wound healing dressings [50, 51]. Some of its chemical properties are its solubility in organic acids [52] and low solubility in water. In order to improve the biological behavior of CTS material, different authors propose the addition of nano-sized structures to increase the physic-chemical and antibacterial properties, such as silver nanoparticles (AgNPs) [53] and gold nanoparticles (AuNPs) [54].

NPs of noble metals are some of the most promising materials, owing to their high surface area and their facility of functionalization or coordination with organic molecules. For example, AuNPs are easily prepared in colloidal solutions. Novel research has been done exploring the potential use of AuNPs as phototherapeutic agents, in the detection and treatment of cancer, in gene therapy and in the transport and selective vectorization of drugs and macromolecules [17, 54]. Otherwise, the AgNPs are widely applied to produce artificial skin, sterilized materials, functional contraceptive devices, antibacterial surgical instruments, bone prostheses, bone coating, surface cleaners, antimicrobial paints, automotive upholstery, food storage, and others [55, 56].

Many synthesis methods have been designed to create blends with metallic NPs and enable the combination and/or synergism of their catalytic, electronic, and optical qualities. Therefore, synthesis of supported gold and silver NPs has attracted lots of attention, in view of their remarkable properties, which depend on the NP size and the amount of each material [57]; they have been used in oxidation reactions, tumor cell targeting and detection, H_2O_2 production and catalytic applications [58–60].

Several studies have been directed to design and understanding the composition and structure of new hybrid polymers. These hybrid materials are made of Au and Ag NPs supported on a polymer grill; the matrix prevents NPs aggregation, provides mechanical backing and keeps biocompatibility. In this area, CTS appears as a unique material with polycationic, chelating, and film forming properties. Additionally, through NPs incorporation its antibacterial effect increases and it can either stimulate or inhibit human cells activity [61].

The AgNPs synthesis allows the production of stable metal NPs. When these NPs are incorporated in CTS electrospun fibers, it is possible to obtain high antimicrobial nanomaterials [62]. This behavior is generated due to the polycationic characteristics of CTS matrix and their interaction with the embedded AgNPs, linked by electrostatic attractions [63]. It has been reported that amine/hydroxyl groups presented in CTS matrix can interact with metal ions, in order to form stable complexes, and it is possible to in situ synthesize metal NPs in CTS solution, with high morphology control [64]. Moreover, AuNPs are also used due to their excellent biocompatibility and especially because it was found that CTS-AuNPs nanocomposites enhance the proliferation of human fibroblasts. This significant enhancement of biocompatibility may be due to the altered surface morphology. The size of the nanometric surface domains could have an impact on cellular responses [65].

The mechanical properties of CTS (e.g. swelling), are not good enough for medical applications; to solve this it was inserted into the structure, a natural synthetic polymer CTS-based, grafted with glycidyl methacrylate (CTS-g-GMA) [66]. This arrangement of polymers provided a new material with better biomedical applications.

Ag and Au NPs show a collective oscillation of their electrons from the conduction band when they interact with a specific electromagnetic field; this property is called surface plasmon resonance (SPR). After all the evidence collected from the interaction between noble metals and natural polymers, the results are the evident success in the aggregation of AuNPs and AgNPs. This behavior was confirmed by UV-vis analyses, where the SPR bands were used to identify the metallic elements. As result, characteristic SPR for the AgNPs was located at 427 nm, while the SPR peak of the AuNPs was located around 530 nm [58], as shown in **Figure 1**.

Fibers obtained by electrospinning have been synthesized modifying the film method, the viscosity did not allow the correct stretched from the solutions, then it was necessary to add to the mixture polyethylene oxide and a surfactant to enhance the viscosity in order to obtain nanometric fibers of the polymer with NPs.

These results are promising, the combined UV-vis spectra from the materials show SPR in 432 nm for AgNPs and 532 nm for AuNPs. Transmission electron microscopy (TEM) allows to observe fibers with several particles inserted in the surface, as presented in **Figure 2**. In this case, is not possible to determine if observed NPs are Ag or Au using only TEM imaging (elemental analyses show the presence of both elements), but the presence of them indicates that the NPs synthesis was successful and the electrospinning method is an option to perform materials with the characteristics to be used in biomedical applications.

The major contribution of this research is that normally both metals, Au and Ag, are reduced chemically by separated and joined after these chemical reductions, nevertheless both nanostructured materials shown above as, films and fibers, underwent the chemical reduction *in situ*.

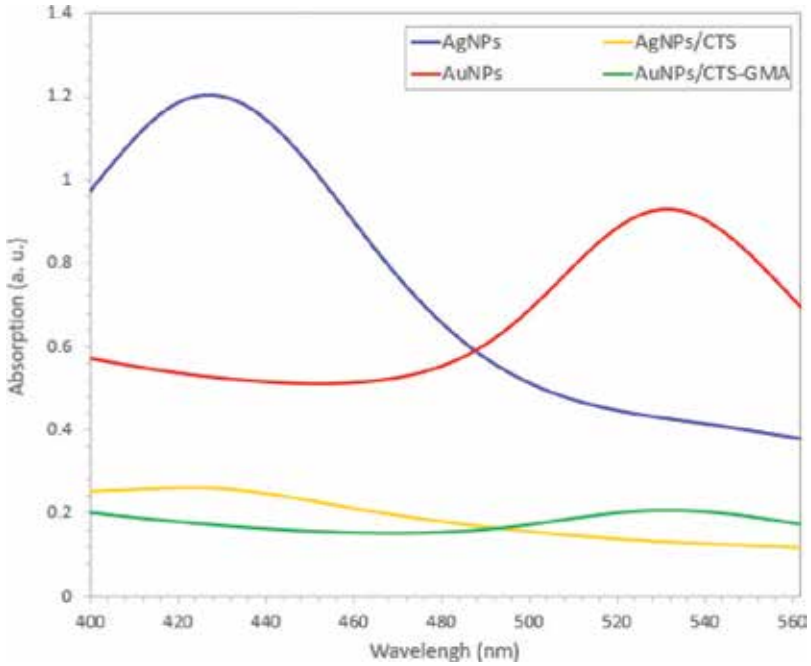

Figure 1. UV-vis absorption spectra of silver nanoparticles (AgNPs), gold nanoparticles (AuNPs), and their corresponding nanomaterials formulated by AgNPs/CTS and AuNPs/CTS-GMA.

Is clear that there are more possibilities for NPs and natural polymers, here we have offered a slight landscape of that, additionally to Au and Ag different metals such as copper (Cu) also could be used in biomedical applications, but the noble metals are a field with an extensive list of contributions elsewhere [67].

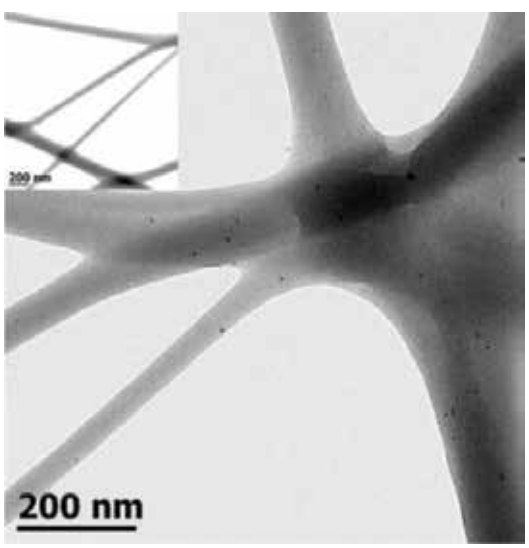

Figure 2. Fibers of CTS with AuNPs and AgNP on the surface of the polymer.

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4. Clinical applications

The novel approach to use nanomaterials in regenerative medicine has established the design of functional tools to simulate, diagnose and stimulate cell growth of tissue or organs [16].

Burn wounds are a critical issue due to the widespread deaths due to the constant bacterial resistance to conventional antibiotics. In this regard, novel nanomaterials such as topic antimicrobial systems have been obtained to produce combined antibacterial/tissue regeneration responses in thermal burns. Luna-Hernández et al. [68] report the use of nanocomposites based on CTS/AgNPs synthesized by *in situ* chemical reduction method, obtaining embedded spherical AgNPs around 7 nm, as presented in **Figure 3**.

In this research, controlled thermal burns produced in rats were treated with nanocomposites with different NPs concentration deposited at wound areas. These results showed the combined antibacterial responses to *S. aureus* and *P. aeruginosa*, depending on NPs concentration and the mesh formation of hydrated chitosan, which allowed bacterial penetration. As a result, significant tissue regeneration was shown in the thermal burns treated with CTS/AgNPs nanocomposites in comparison with untreated one, as presented in **Figure 4**. Also, histological assays showed important tissue regeneration responses in contact with nanocomposites, suggesting the myofibroblasts regeneration and accelerated healing processes compared to uncovered thermal burns.

Chemotherapy and radiation exert their effects by inhibiting tumor cell growth and by blocking tumor reformation. However, some cancer patients present tumor relapse due to cancer

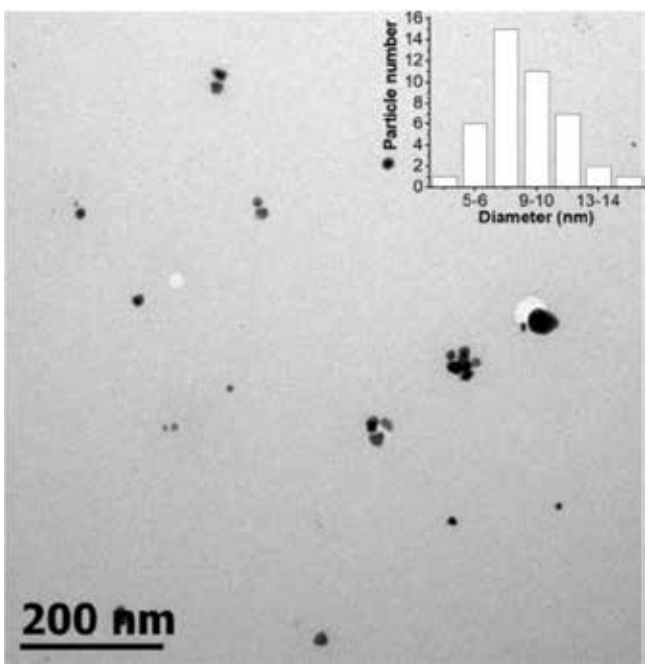

Figure 3. AgNPs synthesized by *in situ* chemical reduction in CTS matrix.

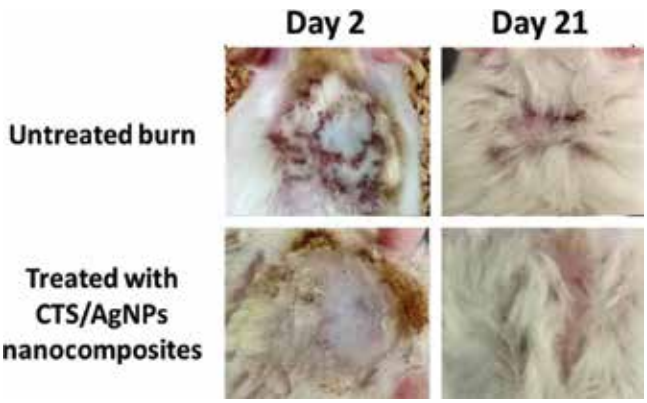

Figure 4. Photographs of controlled thermal burns untreated and treated with CTS/AgNPs nanocomposites.

stem cells, which cannot be killed by these therapies. These cancer stem cells are able to form new colonies and regenerate tumors. It is of great importance to develop new therapeutic approaches to selectively target stem cells. There are novel therapies using NPs to target stem cell-specific markers or signaling pathways [69]. In other hand, glioblastoma multiforme tumors show resistance to radiotherapy and chemotherapy and this is believed to happen due to tumor stem cells. NPs carrying antitumor drugs have to be able to reach the tumor cell, by crossing a series of membranes slide across the blood-brain barrier. For NPs to reach the tumor in a specific way, some strategies have been incorporated like the use of antibodies or peptide molecules which recognize tumor cells antigens to improve the therapeutic efficacy by means of increasing tumor cell uptake and accumulation into the cytoplasm [70].

In other hand, Gilbert and Osterhout suggested the use of NPs from the delivery of chondroitinase ABC in rats, a therapeutic enzyme to treat spinal cord injury in order to cause axon regenerative responses. In this case, the released enzyme from NPs produced digestion of chondroitin sulfate proteoglycans, which are the lesion markers [71]. For spinal cord injuries, it has been reported the use of biocompatible polymer NPs based on poly(lactic-co-glycolic) encapsulated methylprednisolone, which can reduce the possible neurological deficits after spinal cord procedures, considering ultralow drug doses at local delivery [72]. Another route to treat spinal cord disease is by using cerium oxide NPs. In this regard, Das et al. report the anti-oxidant, photocatalytic and biocompatibility behavior of nanomolar concentration of NPs, acting as neuroprotectors without cytotoxic effects [73].

Cancer therapy is a major challenge in order to design alternatives for detection and treatment. In particular, the use of aptasensors is emerging as a novel strategy for cancer detection. Aptasensors described as recognition elements derived from artificial fragments of DNA or RNA, easily synthesized and modified to target as biomarkers, with low immunogenicity and high affinity. In this regard, graphene nanocomposites decorated with metallic NPs obtained from Layer by Layer deposition have been considered a novel tool for specific polypeptides detection [74].

For drug delivery systems, Sahu et al. [75] proposed the use of graphene nanosheets integrated into liposomes as drug delivery vehicles, monitored by NIR light. Some advantages of using NIR light to liposomes detection are their non-toxicity, specificity, and high tissue

penetration. Authors claimed that graphene oxide could act as a light activable switch to trigger drug release from liposomes upon NIR irradiation.

5. Conclusions

In the present chapter, the use of biopolymers-based nanostructures is addressed, including biomaterials and stem cells, bio-nanocomposites, and specific clinical cases where these systems were employed. We addressed the current challenges in the formulation of functional materials based on biopolymers/metal NPs to mimic the cellular behavior of living organisms. It is important to note that material functionality must be improved to synergistic properties, for example, combined antibacterial/tissue regeneration responses, aiming to contribute the specific cell regeneration and avoiding the bacterial colonization. In this sense, the recent trend in nanomaterials development must be focused in the design of functional systems which combine their physic-chemical and biological characteristics, aiming to produce efficient cellular growth and contribute to tissue engineering approaches. We emphasize the future challenges and perspectives in the design of biocompatible and nontoxic nanocomposites with high efficiency as a promoter for tissue regeneration and many other biomedical applications.

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Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

Abbreviations

Ag	Silver
AgNPs	Silver nanoparticles
Au	Gold
CTS	Chitosan

Cu	Copper
DNA	Deoxyribonucleic acid
GMA	Glycidyl methacrylate
H_2O_2	Hydrogen peroxide
MCF7	Epithelial cancer cell line
NIR	Near infrared
NPs	Nanoparticles
RNA	Ribonucleic acid
SPR	Surface plasmon resonance

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Section 3

Matrices for Tissue Regeneration

Chapter 4

Scaffold Biomaterials in Tissue Regeneration in Surgery

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Additional information is available at the end of the chapter

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Abstract

This chapter will focus on the subject of tissue regeneration in a variety of different surgical fields and operations. We will explore the use of acellular dermal matrices, stem cell-based therapies, gene regulation, emerging 3D printing techniques and their potential applications in surgery. Acellular dermal matrices (ADMs) are biological materials derived from human or animal tissue through complicated and expensive decellularisation processes, leading to acellular material that can be used to aid tissue healing. ADMs were first introduced for the treatment of burn injuries, but are now widely used in a variety of surgical fields, including abdominal wall and breast reconstruction. A wide range of materials can be used to produce ADMs, but usually include bovine, porcine or human tissues (e.g., dermis and pericardium). ADMs act as scaffolds onto which human tissue can incorporate, allowing for an innovative, yet a very effective way to aid tissue regeneration. Stem cell therapies also hold promise in aiding tissue regeneration in the coming years and we will also explore techniques that are currently being researched by prominent scientists all across the world. For example, adipose tissue-derived stem cells (ASCs) are a potentially revolutionary therapy in regenerative medicine. We will review the current evidence available and consider the possible clinical applications of ASCs, including their potential to treat ischaemic diseases and their role in healing chronic wounds. ASCs are adult stem cells, which display similar morphology and differentiation properties to adult mesenchymal stem cells (MSCs). The multiple linage pathways displayed by ASCs allows a variety of tissues to be repaired and maintained. Moreover, adipose tissue is abundant, easily accessible and is able to be repeatedly harvested with low morbidity. Previously, autologous fat grafting was more commonly utilised for managing volume defects in reconstructive and plastic surgery; however, recent literature has revealed promising therapeutic effects of ASCs in tissue regeneration. Finally, gene regulation, which holds promise in musculoskeletal diseases, and 3D printed scaffolds that aid neural regeneration will also be discussed in this chapter as emerging, and potentially very promising, tissue regeneration techniques.

Keywords: tissue regeneration, reconstruction, acellular dermal matrix, adipose tissue-derived stem cells, stem cells, gene regulation, 3D printing, bovine pericardium, xenograft, porcine dermal matrix, Veritas, Strattice

1. Introduction

Tissue regeneration is a vast subject, with many different important aspects to consider. From groundbreaking advances in the use of acellular dermal matrices, to the still-evolving stem cell treatments, this chapter provides an overview of the essentials in tissue regeneration science. We will explore the use of acellular dermal matrices, stem cell-based therapies, gene regulation, emerging 3D printing techniques and their potential applications in surgery and provide an overview of wound and tissue healing in general.

2. Acellular dermal matrices

Acellular dermal matrices (ADMs) are biological materials derived from human or animal tissue through complicated and expensive decellularisation processes, leading to acellular material that can be used to aid tissue healing. ADMs were first introduced for the treatment of burn injuries, but are now widely used in a variety of surgical fields, including abdominal wall and breast reconstruction. A wide range of materials can be used to produce ADMs, but usually include bovine, porcine or human tissues (e.g., dermis, pericardium). ADMs act as scaffolds onto which human tissue can incorporate, allowing for an innovative, yet a very effective way to aid tissue regeneration (see **Figure 1**).

First introduced in 1994, a specific acellular dermal matrix (AlloDerm) was used as a dermal substitute in a full thickness burn injury [2]. This overcame the troubling consequences of significant scarring and contracture after the use of split-thickness autografts used for full thickness injuries. A high percentage of 'take' was seen and was assessed using histology and electron microscopy [2]. No specific immune response was seen and this is owing to the processes in which the actual acellular dermal matrices are produced. In addition to the benefits of reduced scarring, contracture and avoidance of immune response, acellular dermal matrices also ensured that any wound of the donor site was avoided—purely due to the fact that the donor site was not necessary! This was particularly important and useful in patients with extensive burns where the donor site availability was limited. All of this led to the increasing popularity of the use of acellular dermal matrices in the treatment of burns and, later on, the introduction of acellular dermal matrices into other surgical fields.

Complex engineering procedures are involved in producing acellular dermal matrices and will also depend on the original type of tissue used. Decellularisation is an essential process and ensures there is no immune reaction once the acellular dermal matrix is introduced into

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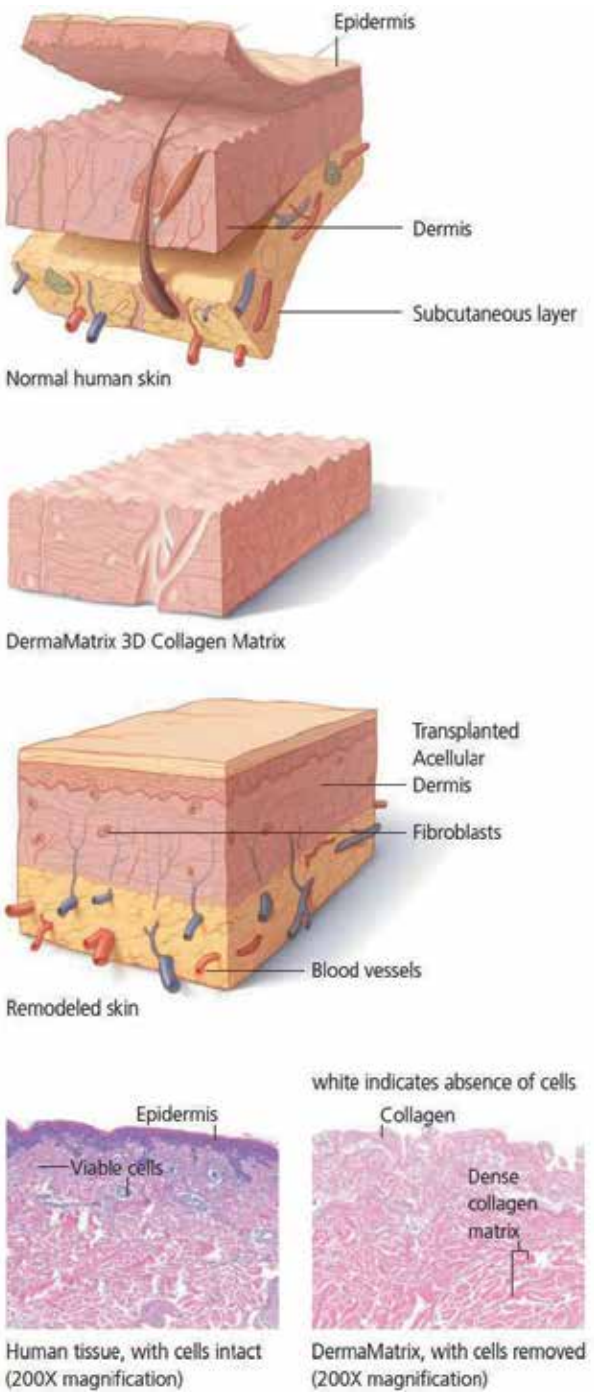

Figure 1. Comparison of DermaMatrix to normal human skin (courtesy of Synthes® [1]).

the recipient's body. All donor cells and antigenic epitopes that have a potential to induce an immune response are removed using a variety of detergents (dependent on a particular type of acellular dermal matrix)—this essentially leaves a scaffold, consisting of collagen, growth factor receptors and vascular channels [3]. Dehydration of the matrix also allows for easier tissue handling and prolonged shelf life [4]. Certain acellular dermal matrices are also terminally sterilised; however, there is no clear evidence whether this provides an advantage [5].

As mentioned above, a variety of different donor tissues can be used in the production of acellular dermal matrices. Commonly, bovine, porcine or human tissues are used, with dermis and pericardium being the most usual types of tissue utilised. A variety of acellular dermal matrices exist at present, some more commonly used examples are listed in **Table 1** [6]:

One of the authors (Chaturvedi) of this chapter has long experience of using the Veritas® acellular dermal matrix, made from bovine pericardium, and has presented this experience in one of the largest series for breast reconstruction [12]. They have found that the advantages of Veritas® included the easy handling and reduction in the incidence of red breast syndrome, as compared to porcine allografts [12].

Acellular dermal matrices act as scaffolds for the recipients' tissues to grow and revascularise upon [2]. Whilst providing nutritional and structural support, acellular dermal matrix integrates into the surrounding tissues and is eventually replaced by functional autologous tissue (see **Figure 2**) [2].

Acellular dermal matrices are used widely in abdominal wall, burn and breast reconstruction. The management of burns with acellular dermal matrices has already been mentioned, with significant benefits of ADM over split thickness skin grafts in terms of donor site sparing, less contracture, scarring and avoidance of immune response. In addition to burns management, acellular dermal matrices were also initially used for tympanic membrane replacement, dural repairs, gingival grafting and, as already mentioned, abdominal wall repair. The use of acellular dermal matrices in these areas gave a start to what is now an increasingly important and prevalent component of both reconstructive and aesthetic surgery.

Breast and plastic surgeons currently actively utilise acellular dermal matrices in a variety of procedures, in particular, implant-based breast reconstruction [5]. Acellular dermal matrices

Name of acellular dermal matrix	Type of acellular dermal matrix
FlexHD® (Ethicon, Somerville, NJ) [7]	Donated human allograft skin
AlloDerm® (LifeCell, Branchburg, NJ) [8]	Donated human allograft skin
Neoform™ (Mentor, Santa Barbara, CA) [9]	Donated human allograft skin
DermaMatrix™ (Synthes, West Chester, PA) [1]	Donated human allograft skin
Permacol™ (Covidien, Boulder, CO) [10]	Porcine dermal implant
Strattice® (LifeCell, Branchburg, NJ) [11]	Porcine dermal implant
Veritas (Baxter, Deerfield, IL) [12]	Bovine pericardium

Table 1. Examples of currently available acellular dermal matrices.

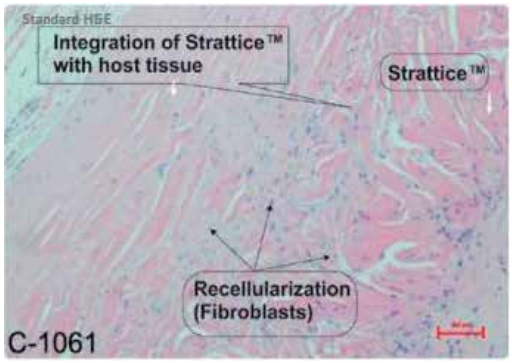

Figure 2. Patient X—Robust recellularisation and remnants of Strattice™ tissue matrix, 31 months post-implantation (courtesy of LifeCell [11]).

have been used in breast surgery since 2001, with many benefits gleaned from their use [5]. Acellular dermal matrices have allowed for immediate breast reconstruction with implants by avoiding the stage of tissue expanders. Mastectomy can be performed with immediate implant based, acellular dermal matrix reconstruction, allowing for immediate results and avoiding a second operation at a later date. Not only do acellular dermal matrices act as scaffolds for tissue regeneration in this case, but also add an additional layer of tissue protection for the foreign body, that is, the implant (see **Figure 3**) [13]. Other examples of applications of acellular dermal matrices in breast surgery include correction of symmastia, incorporation into the upper pole (to decrease surface rippling) and correction of inframammary fold malposition [5]. In addition, acellular dermal matrices are also used in two-stage breast reconstruction procedures with tissue expanders; however, despite advantages, such as faster expansion, improved lower pole projection and better aesthetic shape, the costs are high and need considered prior to individual patient use [5].

New ways of utilising acellular dermal matrices in breast surgery have also been trialled and include use of meshed and fenestrated acellular dermal matrices [14]. This allows for

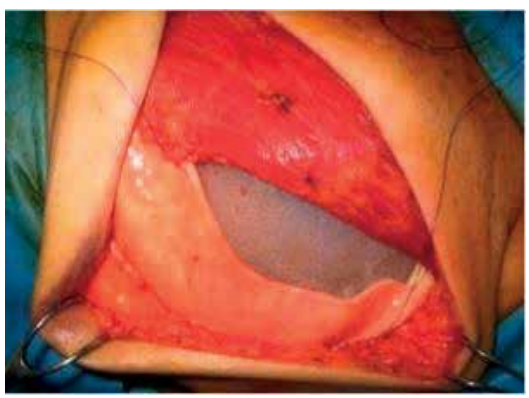

Figure 3. Tissue expander placement into the Pectoralis major and acellular dermal matrix pocket (courtesy of Weichman et al. [12]).

the reduction of costs, with evidence also showing that with fenestrated acellular dermal matrices, the incidence of capsular contractures, infections and seroma formation can be decreased [5].

Complications associated with acellular dermal matrices depend on the type of the acellular dermal matrix used and also the particular procedure it is used for. Breast reconstruction with acellular dermal matrix can cause increased risk of post-operative infection, skin necrosis and post-operative seroma [15]. The correct patient should be identified in order to ensure the risks that are acceptable. Caution needs to be used with obese patients (BMI > 30), simultaneous axillary clearance and smoking history. Radiation will affect any reconstructed breast; however, acellular dermal matrices have, in fact, been shown to reducing the severity of capsular contracture [15].

The use of acellular dermal matrices in abdominal wall reconstruction offers an alternative to a permanent prosthetic mesh and has been in use since mid 2000s [16]. Although some surgeons prefer acellular dermal matrices for abdominal wall reconstructions, concerns have previously been raised regarding the long-term outcomes of acellular dermal matrices as compared to synthetic meshes, with the main worry being the durability. A recent study, however, showed that hernia recurrence rates with acellular dermal matrices were comparable to those done with synthetic mesh—in particular, it was also seen that xenograft acellular dermal matrices led to even lower recurrence rates than human allografts [17]. The question of cost, however, arises again, and synthetic meshes are in fact cheaper than acellular dermal matrices [17].

Outcomes with acellular dermal matrices in breast surgery have already been mentioned (and there is extensive literature for this subject, including a systematic review), but favourable reports have been published on outcomes in pelvic, abdominal, chest wall reconstruction, dural repair, hand surgery, urethral reconstruction and gingival graft procedures, too [6]. Butler et al. successfully used AlloDerm in the reconstruction of large and complex pelvic, chest and abdominal wall defects [18]; however, further studies would be needed in the use of acellular dermal matrices for dural repair (Chaplin et al. successfully used XenoDerm in a porcine model and called it "a nearly ideal dural replacement") [19]. Kim et al. also successfully used acellular dermal matrix for a recurrent first dorsal web space defect, showing excellent cosmetic and functional results [20]. Aichelmann-Reidy et al. showed that acellular dermal matrix could also be a useful substitute in root coverage procedures [21]. Controversies, however, still exist and some studies have shown increased infection rates with ADM-based reconstruction as compared to non-ADM-based reconstruction [22].

Significant costs are also involved when using acellular dermal matrices and remain a topical issue in all fields of surgery. Some situations where costs may be unacceptable have already been considered, for example, with some general surgeons preferencing synthetic meshes in abdominal wall reconstruction due to decreased costs [17]. However, in cases where acellular dermal matrix allows for a two-stage procedure (e.g., implant-based reconstruction with tissue expanders placed during the primary procedure) to be converted into a single-stage procedure (i.e., implant-based ADM reconstruction without the need of tissue expanders), significant savings will be made and this needs to be considered on an individual patient basis.

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3. Mesenchymal stem cells

The exciting field of stem cell therapies has rapidly evolved in order to provide a potential alternative treatment for tissue repair and to enable the regeneration of injured organs. New developments are continually arising from this promising topic of research.

Stem cells are unique in that they are undifferentiated cells that can renew themselves throughout the entire lifespan of an organism. They develop from one common precursor and have the ability to differentiate into multiple cell types with specific functions (see **Figure 4**). Stem cells are characterised by their ability to self-renew over prolonged periods of time [23]. Stem cells that have the potential to repair surgical wounds include mesenchymal stem cells (MSC), embryonic stem cells (ESC) and induced pluripotent stem cells (iPS) [24].

The most commonly utilised stem cells are MSCs, which are derived from adult patients. Autologous mesenchymal stem cells are present in almost all adult tissues including the dermis, periosteum and adipose tissue, solid organs, such as the liver, lungs and spleen and within bone marrow and blood, including from the peripheries, menstruation and the umbilical cord [25].

There has been great enthusiasm within the literature regarding the potential use of stem cells in tissue regeneration over the last decade. The initial focus of research surrounded the clinical applications of embryonic stem cells. However, over the past decade, there has been a move within the scientific community to research the potential applications of mesenchymal

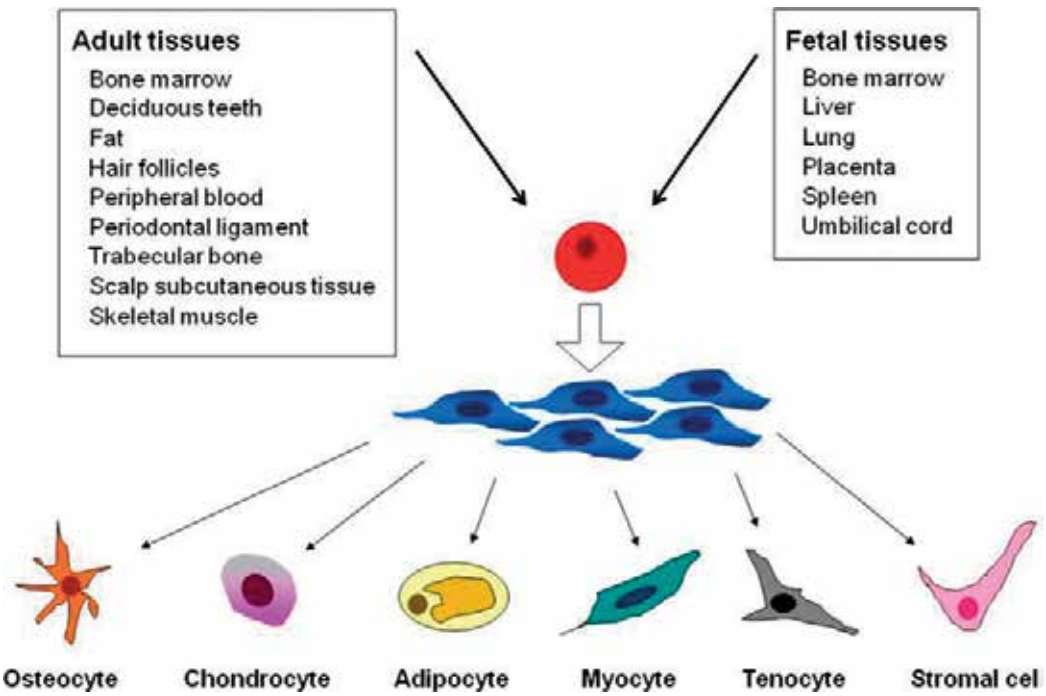

Figure 4. Skeletal regeneration by mesenchymal stem cells: what else (courtesy of Andrades et al. [26]).

stem cells. In comparison to embryonic-derived stem cells, there are less ethical concerns surrounding their cultivation and utilisation [27].

Traditionally MSCs were derived from adult bone marrow using a bone marrow aspirate. However, lately there has been mounting interested in harvesting MSCs from adipose tissue, these are known as adipose-derived stem cells (ASCs). ASCs are of value as they are abundant in supply and easily accessible by means of an excised solid block of tissue or through liposuction techniques [27]. The International Society for Cellular Therapy instituted the following criteria to identify human mesenchymal stem cells (hMSCs) (see **Table 2**) [28].

Adipose tissue is a highly complex tissue derived from mesodermal origin [28]. Its main functions include energy storage, insulation, protection from mechanical injury, endocrine properties and now as a source of multipotent stem cells [29]. It can be classified as brown and white adipose tissue. Thermogenic brown tissue is responsible for energy expenditure and is mostly found in the foetus and new born babies [29]. White adipose tissue is located subcutaneously and intra-abdominally and is responsible for energy storage and insulation (**Figure 5**). White tissue tends to be in abundance and thus renders it a viable long-term option for supply of stem cells [25].

Additionally, subcutaneous adipose tissue can be classified as superficial or deep tissue. The differential potential of ASCs may be altered depending on the location of the harvest. Taranto et el. demonstrated varying stem cell properties within subcutaneous tissues dependant on their location. Adipose tissue yielded from superficial tissues demonstrated increased multipotency [31]. One study has shown that ASCs derived from superficial tissues displayed a slightly higher osteogenic potential than from the deep layer [32]. Previous reports suggest that the yield of ASCs is 100–500 times higher in comparison to bone marrow-derived stem cells [30, 32].

Throughout the literature, there are a number of methods described for the cultivation of MSCs. Naderi et al. describes the isolation and cultivation techniques to obtain ASCs [33]. The adipose tissue is chopped and digested by collagenase and centrifuged in the laboratory. Isolated stem cells are cultivated and subsequently differentiated into a variety of different cell lineages. During pre-clinical trials, ASCs have proven to be very stable under cell culture conditions with a normal haploid karyotype remaining following 100 duplications [34]. ASCs can successfully be cryopreserved whilst maintaining their viability therefore ASCs could be potentially stored prior to use [35].

An extensive volume of research investigating the role and mechanism of action of MSCs in wound healing has been undertaken. Motegi et al. and Fromm-Dornieden et al. recently summarised this into two main categories [28, 34]. These include promoting wound healing through: (I) paracrine actions with nearby cells through the release of growth factors and cytokines and (II) differentiation of cells into resident cells to create a scaffolding to encourage healing. The

1. Proliferation *in vitro* as plastic-adherent cells.

2. Positive expression of CD105, CD73, CD90 and negative expression of the haematopoietic cell surface markers CD45, CD34, and CD14, CD11b and CD79α, or CD19 and HLA-DR.

3. Differentiation into osteoblasts, adipocytes and chondrocytes in culture conditions *in vitro.*

Table 2. Adapted from 'Mesenchymal stem cells: The roles and functions in cutaneous wound healing and tumour growth' [27].

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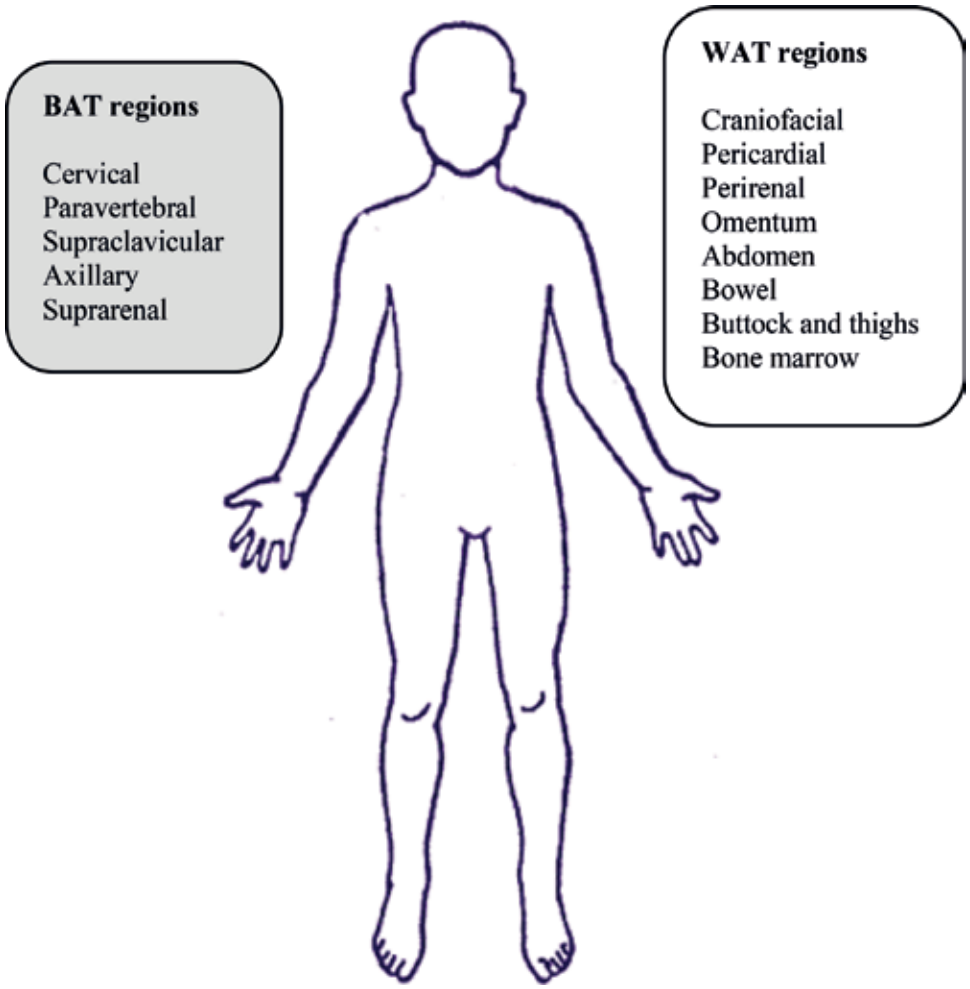

Figure 5. Distribution of brown and white adipose tissue within the human body (courtesy of Kocan et al. [29]).

paracrine mechanisms enable numerous growth factors to be secreted including basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), keratinocytes growth factor (KGF), transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF), which in turn promotes angiogenesis and therefore wound healing [29, 33]. These growth factors are thought to have anti-inflammatory actions, enhancing wound healing by dampening down inflammation at the wound site [29]. Macrophage recruitment is increased. Macrophages are classified as classically activated (M1) and alternatively activated (M2). M2 macrophages have an important role in the progression of wound healing and it is thought that MSCs increase macrophage polarisation in wounds and therefore enhance wound healing [36]. Endothelial cell recruitment is also increased [36]. MSCs have the ability to differentiate into the resident site-specific cells, including, fibroblasts, myofibroblasts, keratinocytes and pericytes [36].

Cells known as pericytes, with similar features to mesenchymal cells, have been discovered within the blood vessels in multiple organs. Crisan et al. described that certain perivascular cells isolated from various organs, including the skin, showed differentiation into multiple lineages

both *in vitro* and *in vivo* [37]. This research surmised that it is likely that blood vessel walls may hold a reserve of mesenchymal-like stem cells that are involved in the repair and neovascularisation of wounds. However, the exact mechanisms and significance remains unknown.

Patient selection for harvesting is an important factor because biologic properties can be affected by systemic disease. Adipose tissue that is extracted from patients with diabetes is inferior to adipose tissue that has not been subjected to systemic disease. In tissue exposed to systemic illness, there is loss of cell differentiation ability, increased levels of failed division and apoptosis and an overall reduction in the levels of growth factors secreted [38].

There are few human clinical trials involving the applications of MSCs and even fewer evaluating the utilisation of adipose cells. The current use of ASCs in clinical practice remains limited to trials. A number of animal model studies have demonstrated the promising possibilities of adipose-derived stem cells and there are a number of small pilot clinical trials, which have been published in the literature recently with many new studies emerging frequently. This exciting data gives promise to the potential clinical applications of ASCs and with new information continuing to evolve, the routine use of stem cells in clinical practice remains a tangible prospect in the near future. This section of the chapter provides up to date evidence and a summary of recent studies involving ASCs.

Nie et al. investigated the mechanisms of action of ASCs in wound healing [39]. ASCs were incorporated into full thickness excisional wounds in both diabetic and non-diabetic rats. The study showed that wound healing was accelerated and time taken to close wounds in both groups was shortened. There was increased re-epithelisation and advanced development of granulation tissue within the wound. Enhanced neovascularisation was also shown due to the increased secretion of angiogenic growth factors.

Park et al. recently investigated the role of allogenic ASCs in the treatment of complex perianal fistulas secondary to Crohn's disease [40]. In this small pilot multicentre, clinical trial participants had complex non-healing perianal fistulas, which had not healed by conventional techniques (surgery or infliximab treatment). The initial group of participants received a smaller dose of ASCs than the second group. At 6-month follow-up, 50% of participants had achieved complete closure of the fistula, which was maintained at the final follow-up at 8 months.

A phase one clinical trial demonstrated the effect of autologous-derived adipose stem cells in patients with severe peripheral arterial disease with chronic non-healing ulcer disease. All participants had non-vascularisable critical limb ischaemia with lower limb rest pain or ulcers and a low ankle systolic oxygen pressure. ASCs were injected intramuscularly into the ischaemic limb with no complications recorded. Most participants showed an increase in trans-cutaneous oxygen pressure and improved ulcer healing [41].

Kim et al. studied the effectiveness of stem cell treatment in patients with chronic non-healing wounds following complications of soft tissue nasal fillers [42]. ASCs were harvested from the patient's subcutaneous adipose tissue. Following preparation in the laboratory, the adipose cell containing solution was injected into the dermis and subcutaneous layer around the wound. All participants experienced enhanced wound healing and at 6 months post treatment all wound sizes were reduced. These results lead the authors to propose that stem cells could be considered in the future for routine use as a treatment of complications of filler injections.

In addition to skin wound healing, there have been advances within the role of stem cells in orthopaedics. A recent study focused on the role ASCs in the repair of meniscal injuries. Toratani et al. created meniscal defects in rabbits and injected autologous stem cells from adipose tissue into half of the subjects [43]. ASCs were found to promote meniscus healing in the rabbit model. This paper offers promise for future clinical uses as a potential new treatment for meniscal injuries subject to further studies.

Stem cells could potentially revolutionise the treatment of chronic heart disease. Atherosclerosis is the leading cause of morbidity and mortality in the developed world with risk factors including diabetes, hypertension, smoking and obesity. Researchers have endeavoured to develop a stem cell-based therapy for the treatments of ischaemic heart disease and cardiac failure. Numerous preclinical studies have demonstrated promising therapeutic benefits using ASCs with the improvement in left ventricular function and reduction in infarct size [44]. However, these successful results have yet to be seen in human trials. The difference in results is thought to be due to the source of stem cells. In animal trials, MSCs were harvested from healthy donors; however, in comparison in the clinical trials, the stem cells were collected from the patient with known atherosclerotic disease and potentially other serious co-morbidities [45]. Further research in this field continues to evolve to in order to create a successful therapy.

4. Gene regulation

Other novel tissue regeneration methods have been trialled in both animal and human studies. For example, genetics is an ever-evolving field when it comes to finding ways and methods of aiding tissue regeneration. Animal studies provide a starting point for future discoveries—for example, Kang et al. investigated tissue regeneration enhancer elements (TREEs), providing evidence that these elements trigger gene expression in injury sites [46]. The authors of this particular study felt that these findings could further be extrapolate in the future to assess their regenerative potential in vertebrate organs [46]. Gene regulation to aid tissue regeneration has been investigated in human studies, too. Recent studies by Finkel et al. and Mendell et al. showed promise in motor neurone diseases, specifically spinal muscular atrophy [47, 48]. Finkel et al. modified promoted increased production of the survival motor neurone (SMN) protein with an antisense oligonucleotide drug and showed that infants with spinal muscular atrophy receiving this drug were more likely to be alive and have improved motor function that the control group [47]. Patients in the Mendell et al. study received adeno-associated viral vector infusion containing DNA coding for SMN; these patients again, survived longer, achieved motor milestones better and had improved motor function than historical cohorts [48]. Musculoskeletal tissue regeneration is a great challenge for scientists and lots of studies have looked into potential options, in addition to the two mentioned already. Padilla et al. discuss a variety of techniques, including blood derived biological drug delivery therapies, which have significant potential for tissue regeneration [49]. For example, platelets release hepatocyte growth factor and stromal cell-derived growth factor 1, both known to be involved in wound healing and proliferation [49]. There is a significant need for further randomised trials and systematic reviews to assess if these therapies could be used routinely for the treatment of musculoskeletal

conditions. These are just examples of how gene regulation can lead to significant changes in tissue regeneration and improved clinical outcomes; and future research will be needed to assess safety of such gene therapies for widespread use.

5. 3D printing

In addition to novel gene regulation techniques, there have also been advancements made in the promising area of three-dimensional (3D) printing for medical needs. 3D printing has revolutionised many aspects of our lives, with its uses and benefits still being tested in medicine. 3D printing has the potential to revolutionise the way we practice medicine and tissue regeneration and transplantation are two fields where opportunities are endless. It is a well-known fact that the need and demand for organ and tissue replacement largely outweighs the supply, even with recently increasing numbers of deceased donors [50]. What if we could eliminate the need for donors and, at the same time, resolve a major issue associated with

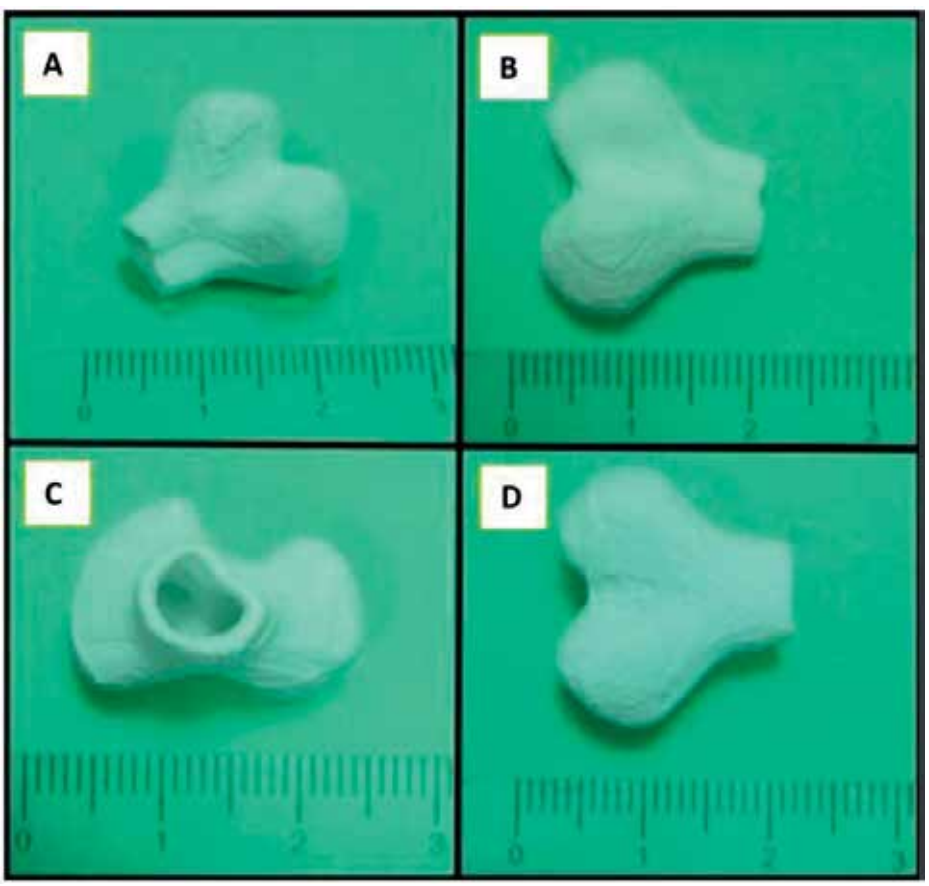

Figure 6. Bone scaffolds generated by selective laser sintering, (A) image of the scaffold, (B) front view, (C) top view and (D) back view of bone scaffold parts, courtesy of Do et al. [50].

organ transplantation – the risk of immune rejection? Do et al. speaks about this in an article about 3D printed scaffolds and their potential applications [51]. The aim would be to create scaffolds that have properties of the native recipient microenvironment and the ability to promote angiogenesis and osteogenesis, and various tissue engineering techniques could be used in order to facilitate this process and this is still a work in progress, albeit an ever-expanding and promising field (see **Figure 6**). Other studies have suggested that 3D scaffolds could also exhibit bactericidal properties, and aid not only tissue regeneration, but also prevent the high risk of infection that comes with any foreign body or implant. Correiaa et al. have shown that silver nanoparticles could be a suitable way to achieve this [52]. The idea of 3D printing has attracted neuroscientists, too, and Zhu et al. hypothesised that the combination of 3D printed scaffolding and low-level light therapy could aid neural regeneration, and favourable results have been achieved in this in vitro neural stem cell study [53]. Further studies will be needed to assess how effective and useful the proposed 3D printing methods for tissue regeneration in humans will actually be.

6. Conclusion

This comprehensive chapter summarised the subject of tissue regeneration in a variety of different fields of surgery. We explored the use of acellular dermal matrices in plastic and reconstructive surgery (e.g., for treatment of burns), breast surgery (e.g., for immediate breast reconstruction after mastectomy) and general surgery (e.g., abdominal wall repair). Stem cell-based therapies were also discussed to reflect the promise they hold in aiding tissue regeneration in the coming years. Particular focus was placed on adipose tissue-derived stem cells and adult mesenchymal stem cells, both of which are a potentially revolutionary therapy in regenerative medicine. Finally, we discussed potential future benefits of using three-dimensional printed scaffolds and gene regulation—both of these fields are currently being investigated by scientists across the world to discover how best to adapt these techniques in day-to-day clinical practice.

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Chapter 5

The Role of Extracellular Matrix in Tissue Regeneration

Dwi Liliek Kusindarta and Hevi Wihadmadyatami

Additional information is available at the end of the chapter

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Abstract

Extracellular matrix (ECM) is an extensive molecule network composed of three major components: protein, glycosaminoglycan, and glycoconjugate. ECM components, as well as cell adhesion receptors, interact with each other forming a complex network into which cells reside in all tissues and organs. Cell surface receptors transduce signals into cells from ECM, which regulate diverse cellular functions, such as survival, growth, proliferation, migration, differentiation, and some vital role in maintaining cells homeostasis. This chapter emphasizes the complex of ECM structure to provide a better understanding of its dynamic structural and functional characterization and multipotency. In this chapter the implications of ECM in tissue remodeling are mainly discuss on the neuronal regeneration and wound healing mechanism in the presence of human umbilical mesenchymal conditioned medium (HU-MSCM).

Keywords: extracellular matrix, ECM components, HU-MSCM, wound healing, neuron regeneration

1. Introduction

ECM is a non-cellular structure that regulates almost all of the cellular functions. ECM is a highly dynamic structural network that continuously undergoes remodeling mediated by several matrix-degrading enzymes during normal and pathological conditions. Deregulation of ECM composition and structure has an association with the development and progression of several physiological and pathologic conditions. In this chapter, we describe the structure and function of ECM, also the role of ECM on the wound healing mechanism and neuronal regeneration in the central nervous system (CNS) and peripheral nervous system (PNS).

2. The structure and function of extracellular matrix

An essential part of the holding capacity of tissues is the extracellular area. The extracellular region is primarily occupied by a complicated network of macromolecules constituent called as extracellular matrix (ECM). The composition of ECM is varied, depends on the species and also developing or ground molecules (**Figures 1** and **2**). Commonly, the ECM is composed of three major classes of biomolecules; there are glycosaminoglycans (GAGs), linked to a protein known as the proteoglycans, and also fibrous proteins, including collagen, elastin, fibronectin, vitronectin, and laminin.

In addition, connective tissue (**Figure 3**) is also composed of the matrix of ECM. One of the essential components of connective tissue is fibroblasts and ground substance. Ground substance is a mixing complex between GAGs, proteoglycans, and glycoproteins (mainly laminin and fibronectin). In most connective tissues, the matrix constituents are secreted by fibroblasts, but in several certain specialized types of connective tissues, like cartilage and bone, these components are secreted by chondroblasts and osteoblasts (**Table 1**).

In general, all the cells need to attach to the extracellular matrix to grow and multiply. Extracellular matrix provides support and anchorage for the shape of the cells, regulates and determines cells dynamic and behavior including cell survival, cell proliferation, cell polarity, cell differentiation, cell adhesion, and cell migration. Moreover, the ECM, also gives the mechanical support for tissues and is involved in the growth mechanism, regenerative, and healing processes.

2.1. Glycosaminoglycan (GAGs)

GAGs are unbranched chains of polysaccharides; GAGs are composed of repeating disaccharide units and are heterogeneous groups in negatively charged polysaccharide chains that are covalently linked to proteins to form proteoglycan molecules. The name GAGs is because in

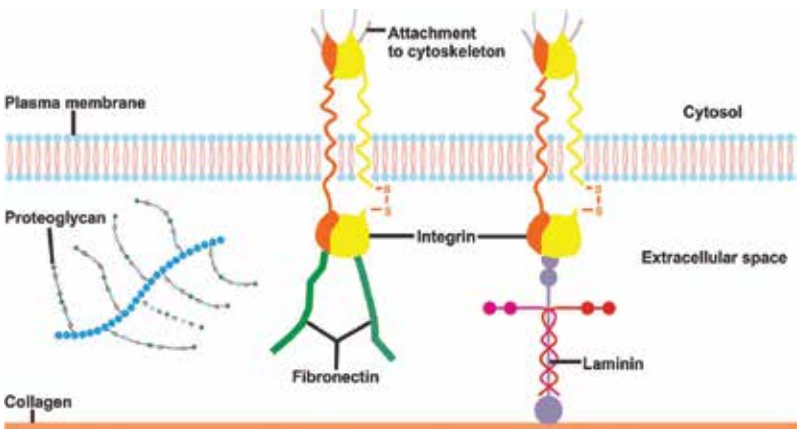

Figure 1. The structure of the extracellular matrix. The ECM mainly contained collagen fibers. There are also some glycoproteins as an adhesion molecule, such as integrin family fibronectin and laminin, which conduct cell attachments to the ECM by binding to collagen in the ECM and integrin. The intracellular part of integrin highly associated with the cytoskeleton thus may promote to anchoring the cell. In the end, there are various proteoglycans in the ECM that act as primary proteins and are profoundly modified by the addition of sugars.

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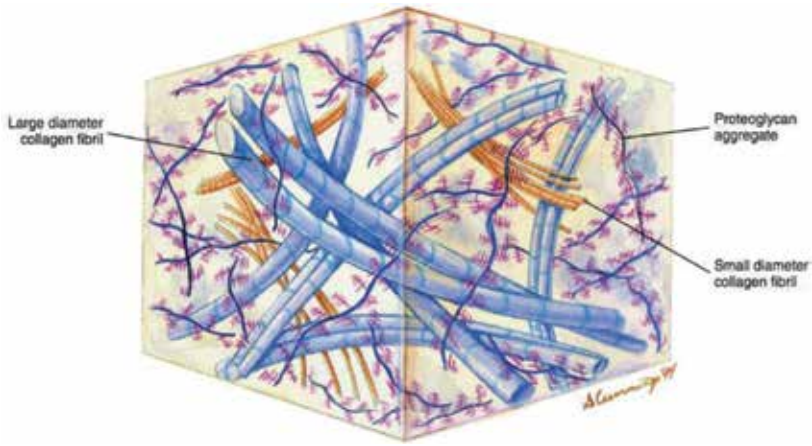

Figure 2. The extracellular matrix of hyaline cartilage found in abundant collagen fibril and proteoglycan aggregates. The chemical analysis of the ground substance reveals that it contains a few glycoproteins and a high concentration of three types of glycosaminoglycans: hyaluronic acid, chondroitin sulfate, and keratan sulfate. Adapted from Crammer and Bakkum [1].

this polysaccharide, one of the two sugars in a repetitive disaccharide is always an amino sugar such as N-acetylglucosamine or N-acetylgalactosamine [3]. The second sugar of GAGs usually is the uronic acid like glucuronic or iduronate. GAG molecules are negatively charged, because there are sulfate or carboxyl groups in most of the sugar. The five main groups of GAGs are differentiated based on the sugar type including (1) hyaluronan or hyaluronic acid, (2) chondroitin sulfate, (3) dermatan sulfate, (4) heparan sulfate, and (5) keratin sulfate. Hyaluronan is the simplest GAGs. Hyaluronan does not contain sulfate sugars; all disaccharides units are the same, and the chain length is extensively big (thousands of sugar monomers). The hyaluronan

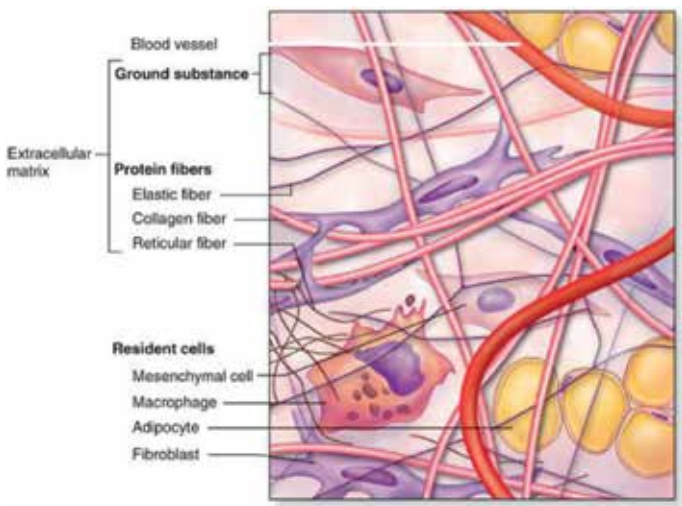

Figure 3. The components of connective tissue. In addition to the extracellular matrix, connective tissues are characterized by a lot of cells, mainly the fibroblasts, all of which are surrounded by the ground substance. Modified from Mescher [2]. *Source:* Michael W. King: Integrative Medical Biochemistry Examination and Board Review, www.accesspharmacy.com

Cell Type	Function	Distribution	Characteristics
Fibroblast	Synthesize and secrete collagen, elastic fibers, reticular fiber, and proteoglycan (among other molecules) Support ligaments, tendons, bone, skin, blood vessels, and basement membranes	Throughout all loose and dense connective tissue	Flat, stellate cells with dark, ovoid, staining nuclei, and one or more nucleoli Microscopically may appear to be of different shapes because of the plane of sectioning
Chondroblast	Synthesize and secrete extracellular matrix of cartilage (collagen, elastic fiber and glycosaminoglycans) Support articular cartilage	Present in hyaline cartilage of articulations and fibrocartilage of intravertebral discs Found also in elastic cartilage	Metabolically active with large vesicular nuclei and prominent nucleoli Cytoplasma pale and vacuolated because of high content of lipid and glycogen
Osteoblast	Synthesize and secrete extracellular matrix of bone	In bone	Basophilic cytoplasma resulting from presence of a large amount of rough endoplasmic reticulum that produce glycosaminoglycan and glycoprotein
Myofibroblast	Synthesize and secrete components of extracellular matrix Capable of contractility	In blood vessels and skin throughout the body	Resemble fibroblast under light microscopy but ultrastructurally contain actin filaments for contraction

Modified from Crammer and Bakkum [1].

Table 1. The cells of connective tissue.

is not connected covalently to some core proteins. Proteoglycans are composed of GAG chains that are covalently linked to the core protein and considered to have a significant role in chemical signaling among cells (**Figure 4**).

2.2. Collagen

Collagen is a major abundant fibrous protein in the extracellular matrix. Collagens, which constitute the primary structural element of the ECM, provide tensile strength, regulate cell adhesion, support chemotaxis and migration, and direct tissue development [4]. Recently, there have been already described 28 types of collagen. The main types of collagen found in connective tissues are types I, II, III, V, and XI.

Collagen polypeptide chains are synthesized on membrane-bound ribosomes and fed into the lumen of the endoplasmic reticulum as large precursors, called the pro-α chains. Each pro-α chain then joins the other two to form a hydrogen-bond, triple-stranded hydrogen molecule known as a procollagen. After secretion, the fibrillar procollagen molecule divides to become collagen molecules, which converge into fibrils [5].

2.3. Fibronectin

Fibronectin is an extracellular protein that makes cells adhere to the matrix. Fibronectin is considered as a large glycoprotein found in all vertebrates. Fibronectin usually exists as a

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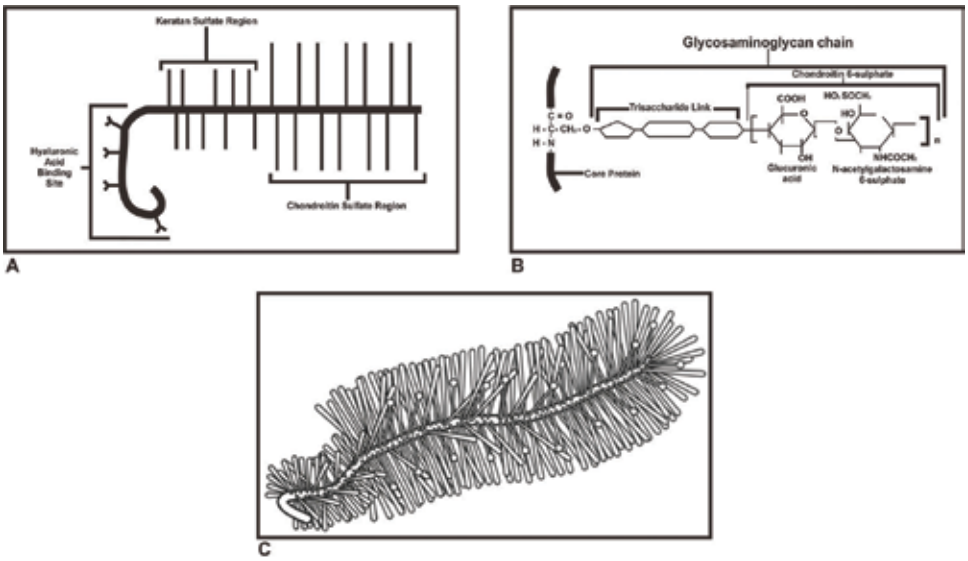

Figure 4. The structure of glycosaminoglycan (A) structure of a proteoglycan monomer. Several glycosaminoglycan chains (chondroitin sulfphate and keratan sulfate) attached to a core protein. The protein molecule can connect to a long hyaluronic acid molecule to help form a proteoglycan aggregate. (B) An example of an individual glycosaminoglycan chain, in this case, chondroitin 6-sulphate, and its attachment to the core protein. (C) The morphological of a proteoglycan monomer. (A and B) Adapted from courtesy Dino Juarez, National University of health sciences; (C) Adapted from Crammer and Bakkum [1].

dimer composed of two nearly identical ~250 kDa subunits linked covalently near the C-terminal by a pair of disulfide bonds at one end side. Fibronectin is a ligand member of the integrin receptor family. Integrins are structurally and functionally related to the cell surface as heterodimeric receptors that link the ECM with the intracellular cytoskeleton.

The primary type of fibronectin is known as type III fibronectin replica (cylinder), which binds to integrins. This model has a length of about 90 amino acids. Fibronectin appears in a soluble and fibrillar form. There are two others fibronectin isoforms, which are fibronectin type I (hexagon) and fibronectin type II (square) [6]. Fibronectin is not only crucial for attaching cells to matrices but also to guiding cell migration in vertebrate embryos. Fibronectin has many functions, which allow it to interact with many extracellular substances, such as collagen, fibrin and heparin, and with specific membrane receptors in responsive cells.

3. Tissue regeneration

Extracellular matrix is the primary factor required in the process of forming a new network and tissue. Along with the development found, many different factors can trigger the growth of ECM or used to create a synthetic ECM. Currently, ECM is involved in various mechanisms such as wound healing with or without the involvement of mesenchymal conditioned medium and neuronal regeneration capability associated with pathologic and/or neurodegenerative disease.

The process of wound healing is strongly influenced by the role of migration and proliferation of fibroblasts in the injury site. Indeed fibroblast is one part of ECM. The proliferation of fibroblasts determines the outcome of wound healing. Fibroblasts will produce collagen that will link to the wound, and fibroblasts will also affect the process of reepithelialization that will close the wound. Fibroblasts will produce type III collagen during proliferation and facilitate wound closure. During proliferation stage, fibroblasts proliferation activity is higher due to the presence of TGF-stimulated fibroblasts to secrete bFGF. The higher number of fibroblasts also induces increasing of collagen synthesis. Collagen fiber is the major protein secreted by fibroblast, composed of extracellular matrix to replace wound tissue strength and function. Collagen fibers deposition was significant on 8–10 days after injury. The number of fibroblasts increases significantly, in correlation with the presence of an abundance of bFGF on 8–10 days after wounding.

Mesenchymal stem cell conditioned medium (MSCM) can be defined as secreted factor that referred to as secretome, microvesicle, or exosome without the stem cells which may found in the medium where the stem cells are growing. The use of MSCM as cell-free therapy has more significant advantages in comparison to the use of stem cells, mainly to avoid the need of HLA matching between donor and recipient as a consequence to decrease the chance of transplant rejection. Additionally, MSCM is more easy to produce and save in large quantity. The presence of human umbilical mesenchymal conditioned medium (HU-MSCM), will accelerate curing of the acute and chronic incision and/or burn wound by increasing the number of myofibroblasts and encouraging the expression of VEGF, TGF, bFGF, and also PDGF to promote wound closure.

Recently, it has been mentioned that widespread neuronal cell death in the neocortex and hippocampus is an ineluctable concomitant of brain aging caused by diseases and injuries. However, recent studies suggest that neuron death also occurs in functional aging and it seems in related to an impairment of neocortical and hippocampal functions during aging processes. Data from WHO and Alzheimer report show increasing number of people suffering from dementia along with aging. Profoundly understanding the role of extracellular matrix (ECM) in influencing neurogenesis has presented novel strategies for tissue regeneration (**Figure 5**).

Central nervous system injury because of stroke vascular and amyloid plaque accumulation as the effect of Alzheimer's diseases may cause the disturbance astrocytes, fibroblasts, and oligodendrocyte precursors cell proliferation which may form a glial scar [8, 9]. Within this glial scar, upregulated proteoglycans like CSPGs and changes in sulfation patterns within the ECM result in the building of regeneration inhibition [10].

To solve the problem, some manipulation on the intrinsic extracellular matrix by using traditional herb such as ***Ocimum sanctum*** extract was already done. In the in vivo and in vitro model using human brain microvascular endothelial cells (HBMECs) which mimics blood-brain barrier, the treatment of the extract may promote the cell proliferation on the hippocampus area and HBMECs in the condition upregulation of choline acetyltransferase (ChAT) enzyme [11, 12]. In addition, there is also a chance to use nanometer-sized scaffolds in the presence of other substrates such as vascular endothelial growth factor or hyaluronic acid with laminin. This scaffold may conduct a way to the regenerative capacity and functional recovery of the CNS to reconstruct formed cavities and reconnect neuronal processes. Thus, the artificial scaffold functions to enhance the communication between cells, allowing for

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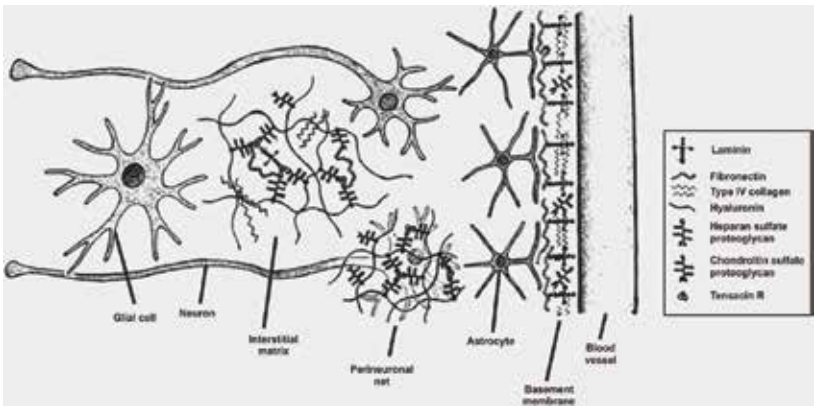

Figure 5. Microscopic anatomy of the extracellular matrix within the central nervous system (CNS). The three major compartments of the extracellular matrix in the CNS are the basement membrane, perineuronal net, and neuronal interstitial matrix. The basement membrane is found surrounding cerebral blood vessels, the perineuronal net is a dense matrix immediately surrounding neuronal cell bodies and dendrites, and the neuronal interstitial matrix occupies the space between neurons and glial cells. Adapted from Lau et al. [7].

improvement in proliferation, migration, and differentiation [13–15]. This evidence gives a new chance in the involvement of HU-MSCM to promote and recover from neuronal injury.

In addition, on the peripheral nerve injury, there is a chance to use scaffold by a chemical decellularization process, acellular nerve allografting that eliminates the antigens responsible for allograft rejection and maintains most of the ECM components, which can effectively guide and enhance nerve regeneration. In the field of tissue engineering by an in vivo model, a lot of successful carriers and matrices have been employed as a scaffold to promote direct axonal growth on peripheral nerve injury [16].

In conclusion, the extracellular matrix is the primary factor required in the process of forming a new network and tissue. Along with the development found, many different factors that can trigger the growth of ECM are used to create a synthetic ECM. Recently, ECM is involved in various mechanisms such as wound healing with or without the involvement of mesenchymal conditioned medium and neuronal regeneration capability associated with pathologic and or neurodegenerative disease. In addition, on the peripheral nerve injury, there is a chance to use scaffold by a chemical decellularization process, acellular nerve allografting to eliminate the antigens responsible for allograft rejection and maintain most of the ECM components, which can effectively guide and enhance nerve regeneration. In the field of tissue engineering by an in vivo model, significant progress on matrices development have been utilized as a scaffold to promote direct axonal growth on peripheral nerve injury.

Conflict of interest

The authors declare there is no conflict of interest.

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Section 4

Stem Cells for Tissue Regeneration

Chapter 6

Synovia-Derived Mesenchymal Stem Cell Application in Musculoskeletal Injuries: A Review

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Additional information is available at the end of the chapter

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Abstract

Musculoskeletal injuries impact millions of people globally and affect their health and well-being as well as of their companion and athletic animals. Soft-tissue injuries represent almost half of these and are associated with unorganized scar tissue formation and long time-depending healing processes. Cell-based therapeutic strategies have been developed in the past decades aiming at the treatment and reversion of such disorders. Stem cells are fairly appealing in the field, being a responsive undifferentiated population, with ability to self-renew and differentiate into different lineages. Mesenchymal stem cells (MSCs) can be obtained from several adult tissues, including the synovial membrane. Synovia-derived MSCs can be found in individuals of any age and are associated to intrinsic regenerative processes, through both paracrine and cell-to-cell interactions, thus, contributing to hosts' healing capacity. Studies have demonstrated the potential benefit of synovia-derived MSCs in these regenerative processes in both human and veterinary medicine. The purpose of this chapter is to review the literature regarding SM-MSC therapies applied to musculoskeletal disorders, in both human and veterinary medicine.

Keywords: musculoskeletal injuries, regenerative medicine, cell-based therapies, mesenchymal stem cells, synovial membrane

1. Introduction

Musculoskeletal injuries represent a major health issue worldwide, compromising society's health and well-being [1]. In osteoarticular and skeletal muscle clinical injuries, tissue self-healing mechanisms are often insufficient and associated to scar tissue formation and long-term

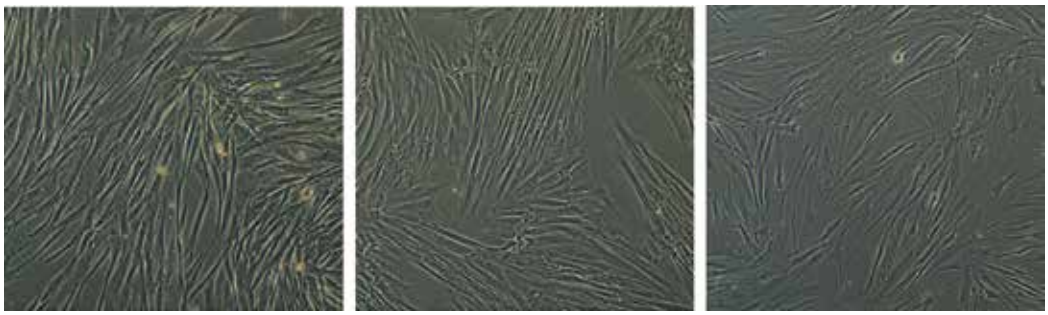

Figure 1. Synovial membrane-derived MSC can be obtained from different species. From the left to the right: canine, equine, and human. Images of cultured cells were obtained from the work developed within our research group.

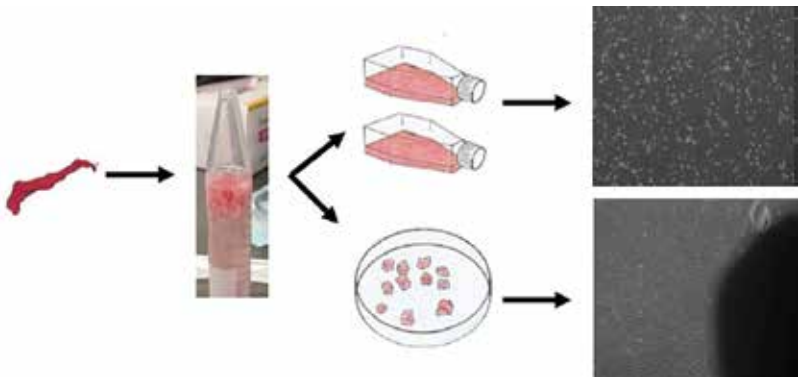

Figure 2. Synovial membrane-derived MSCs obtained from enzymatic digestion and explants technique; images of isolated cells were obtained from the work developed within our research group.

healing processes [2–5]. Different treatment techniques have been developed in the past years, but until now, no ideal regenerative treatment approach has yet been established [2, 4].

The purpose of this chapter is to review on the available literature regarding synovial membrane-derived MSC therapies applied to musculoskeletal disorders, both in human and veterinary medicine. **Figure 1** illustrates synovial membrane-derived MSC from three different species: canine, equine, and human.

MSCs can be obtained from the synovial membrane tissue through two different procedures: enzymatic digestion and explants technique, both illustrated in **Figure 2**.

We will address the musculoskeletal injuries and intrinsic repair mechanism and MSC sources applicable for its treatment, focusing on the advantages of synovial membrane-derived MSCs.

2. Musculoskeletal injuries

2.1. Osteoarticular disorders and regeneration physiology

The articular cartilage (AC) is a thin connective tissue layer that covers the bone extremities of the joint [4]. The AC presents a notable matrix structure organization [6], a limited number of

chondrocytes [7], and a rich water content [8]. The most important biomechanical functions of the AC include weight bearing and a smooth distribution of forces to the adjacent subchondral bone, providing nonfrictional motion of joints [4, 8]. The AC is divided into three layers. The most superficial one is thin, with a smooth surface. In this layer, the collagen fibers are aligned parallel to the tissue surface. In the middle, the articular cartilage is constituted by larger collagen fibers, with a nonparallel organization structure. The deep zone has a parallel alignment of the collagen fibers, vertically to the tissue surface [8]. The unique matrix structure, rich in collagen fibers, proteoglycans, and interstitial fiber, provides a viscoelastic environment that allows the AC to support its biomechanical functions [8]. It has been well established that the AC has limited self-healing capacities [3–5, 7–15] due to its intrinsic characteristics, namely its avascular nature, limited number of resident stem cells, and unique matrix organization [4]. Partial defects on mature cartilage do not heal spontaneously. On the other hand, complete defects are associated with the formation of fibrocartilage, which presents inferior mechanical characteristics [4]. Injuries affecting both, AC and subchondral bone, named osteochondral lesions, often evolve to secondary osteoarthritis (OA) [8, 9]. OA is a syndrome, characterized by AC degeneration, matrix loss, fissure formation, culminating with defects on the cartilage surface, and impacting on surrounding articular tissues, such as the subchondral bone, joint ligaments, the synovial membrane (SM), and periarticular muscle tissue [16], culminating with joint dysfunction and severe pain [9]. OA is one of the most frequent diseases affecting individuals worldwide, thus representing a major impact on the society's health [17]. Many other diseases culminate in OA, if not diagnosed early and treated, such as osteochondritis dissecans, affecting specially teenagers and young adults [18].

Chondrocytes are highly specialized cells, responsible for the production and maintenance of healthy cartilage matrix [19, 20]. However, these cells are particularly differentiated, with poor migration and proliferation abilities; thus, treatment represents a problematic challenge [17]. Several surgical treatment approaches have been developed in the past years. However, they all have inherent problems, impacting on patients' long-term healing process [21]. Surgical procedures that stimulate the bone marrow (BM), such as abrasion, distraction, drilling, and microfractures, are said to promote chondrogenesis phenomena, by inducing the BM mesenchymal stem cells (MSCs) from the subchondral bone. However, in most cases, these techniques lead to the formation of fibrocartilaginous tissue, instead of hyaline cartilage, probably due to an overloading of the BM and a small number of MSCs available, and the repaired cartilage often degenerates in the long term [3–5, 11]. Alternative regenerative approaches, regarding cartilage tissue engineering, are being developed, in order to overcome these disadvantages. Mosaicplasty is characterized by the transplantation of various small autologous osteochondral grafts to the injured joint site [13]. This procedure, however, is not suitable for OA patients or suffering from rheumatoid arthritis (RA), as chondrocytes in these patients have different biological properties [14]. This procedure promotes a short-term relief on patient's symptoms but fails to repair the damaged tissue and hyaline cartilage [4]. RA is a systemic autoimmune disease, characterized by a continuous inflammation phenomenon, a result of an intrinsic imbalance, culminating in a major synovial hyperplasia, bone, and cartilage damage [15]. Treatments involving artificial prosthesis are quite invasive and lifetime limited [9], as well as the mosaicplasty treatment technique is invasive and causes damages to the donor site [13] and fails to restore functional, as well as, phenotypically stable hyaline cartilage [4].

Autologous chondrocyte transplantation (ACI) is a cell-based technique that consists of harvesting chondrocytes from a nonweight bearing joint, first reported by Brittberg et al. [9]. Chondrocytes are expanded *in vitro* in a monolayer culture and then implanted in the lesion site. Despite the small amounts of donor cartilage used, it is necessary to minimize the invasiveness of the technique [13]. During the *in vitro* expansion period, many chondrocytes dedifferentiate and become unsuited to produce stable hyaline cartilage, thus impacting the final clinical outcome [4, 18]. Further, an uneven distribution of the transplanted chondrocytes at the lesion site is very common, as well as the diffusion of the cells from the cartilage defect [8]. To overcome these difficulties, transplantation of tissue-engineered cartilage was developed, evolving *ex-vivo* techniques, however with short-term successful results [18], in part due to intrinsic characteristics of the AC, as its antiadherent properties, which do not facilitate the integration of repaired tissue into the adjacent cartilage tissue [8].

2.2. Skeletal muscle injuries and repair mechanisms

Musculoskeletal disorders also impair the life and well-being of millions of individuals. They are usually characterized by long and incomplete healing processes that culminate into permanent musculoskeletal lesions [1].

Regarding the muscular tissue, in specific, the skeletal muscle, its constitution includes syncytial fibers that are characterized by the presence of a peripheral, postmitotic myonuclei [22]. Under experimental conditions, the skeletal muscle presents notable regeneration ability. Concerning clinical disorders, injuries or ischemia results in considerable tissue loss, that is, generally, not replaced [23]. In an adult, the intrinsic healing capacity of the skeletal muscle tissue relies on the presence of a resident, mononuclear, undifferentiated cell population, known as satellite cells (SCs) [22]. These cells are located between the sarcolemma of myofibers and the basal lamina [1, 24] and have the ability to migrate considerable distances, within the muscle tissue [23]. In a mature, healthy musculoskeletal tissue, SCs are predominantly on a mitotically quiescent state and respond to environmental signaling [22]. It is well established that microenvironmental signals are responsible for gene reprograming and cell phenotype changes [25]. Those signals, resulting from biophysical phenomena, such as growth, injuries, or weight bearing, induce existing SC to proliferate, differentiate, and fuse to existing muscle fibers, thus, mediating postnatal muscle regeneration [22, 26]. These environmental signals comprehend the release of growth factors from the impaired muscle fibers [1, 23], more accurately, myogenic regulatory factors (MRF), which are MyoD, Myo5, myogenin, and MRF4 [22]. The first two growth factors have a more active role during the embryonic development of the skeletal muscle lineage. After division, the SCs become myoblasts, which undergo a terminal differentiation process and fusion to the preexisting muscle fibers. Myogenin is responsible for promoting the terminal differentiation process and fusion [22]. MyoD also plays an important role by promoting the beginning of the proliferation phase of the SC. The absence of MyoD implies a cycle where SCs suffer several division rounds but return to a quiescent state [27]. On the other hand, lack of myogenin causes a severe deficit in the muscle tissue differentiation, resulting in the formation of unfunctional muscle fibers [28]. Thus, satellite cells recapitulate the MRF expression from the embryonic stage, during muscle repair processes. But, when in a quiescent state, SCs do not express detectable levels of MRF [1].

Primary myopathies are characterized by a progressive atrophy of skeletal muscle fibers, thus resulting in deterioration, and compromising movements [2]. As the intrinsic repair ability of the mature skeletal muscle is limited, and pharmacology suppression of the inflammatory and immune response only provides a mild and finite effect, alternative cellular therapies have been developed, aiming at promoting the healing process [1, 2, 22]. Myoblasts would be an obvious choice, due to their role in the muscle repair mechanism. However, they are poorly expandable *in vitro* and undergo senescence quite easily [2, 22]. It is reported that about 90% of the transplanted myoblast cells die within the first hours [25]. Most genetic muscular disorder defects lie in the protein binding between the extracellular matrix and the cytoskeleton of the muscle cell, thus resulting in mechanical stress and continuous contraction movements, leading to muscle degeneration, and consequent tissue loss [2]. The Duchenne muscular dystrophy (DMD) is one of the most common genetic disorders in children. It is characterized by the lack of dystrophin at the muscle fiber sarcolemma. This disorder results in progressive and irreversible muscle degeneration and consequent death [1, 22]. Regarding these genetic disorders, myoblasts exist in small number and are not easily recovered in muscle biopsies [22]. Moreover, in the earlier stages of the disease, SCs divide to form myoblasts that fuse to the existing muscle fibers. However, those SCs transport the exact same genetic defect as the other muscle cells they are replacing. Thus, they will eventually die too [2].

3. MSC sources applicable for musculoskeletal regeneration

Regenerative medicine approaches regarding stem cell therapies have been developed in the past decades as a promising strategy, focusing primarily on immune/anti-inflammatory modulation [15] and cancer treatment [29]. Furthermore, their potential has been employed in cartilage [30] and skeletal muscle repair [1], the latter in a more immature state of development.

MSCs represent a fair candidate to innovative therapies because of their intrinsic unique abilities. MSCs were first harvested from the bone marrow by Friedenstein in 1976 [29, 31, 32], but now their presence is well established in virtually all postnatal tissues [5, 31–34], being involved in the tissue growth and homeostasis [31]. They have since been isolated from different adult tissues [30–32, 35, 36], such as fat, bone marrow, bone, cartilage [6], periosteum [32], nervous tissues, tendon, ligament, epithelium, SM, lung, peripheral blood, skeletal muscle, and nonadult tissues, such as amniotic fluid, placenta, and umbilical cord blood and stroma [29].

MSCs are plastic-adherent cell, fibroblast-like [29, 36], able to self-renew [32, 37]. They are characterized by an extensive proliferation ability in culture and have the potential to differentiate *in vitro* into different lineages [5, 32, 33, 36], including adipogenic, chondrogenic, osteogenic [6, 29, 31, 35], myogenic [6, 31, 35, 37], and neurogenic [6, 37].

MSCs' unique characteristics explain the interest of application on the development of regenerative cell therapies: ease of isolation, high expansion rates *in vitro*, low immunogenicity, and multipotency [4]. However, MSCs' definition and characterization still represent a challenge in the actual days.

According to the International Society of Cellular Therapy (ISCT), MSCs are characterized based on three important criteria: cell adherence, when cultured in standard conditions [38, 39], expression of specific cell surface markers (cluster of differentiation (CD)73, CD90, and CD105) and negative to others (CD45, CD34, CD11b, CD79a, and HLA-DR), and differentiation potential *in vitro* into multiple lineages: osteogenic, adipogenic, and chondrogenic, in defined culture conditions [38, 39]. They are furthermore characterized with respect to colony-forming unit fibroblast (CFU-F) [38, 40]. General consensus has not yet been established regarding specific cell surface markers [4]. It is not possible to characterize these cells only by the use of one specific cell marker [41]. Different protocols are applied to compare MSCs from different sources, always respecting the minimal criteria proposed by the ISCT. Nevertheless, there is no uniformity among the different characterization protocols [4]. The parallel expression or exclusion of several cell surface markers, associated with other ISCT criteria, is a frequent approach to MSC identification [41]. They share nonhematopoietic cell surface markers, such as CD29, CD44, CD73, CD90, and CD105 and human lymphocyte antigen (HLA) [29]; CD9, CD44, CD54, CD90, CD166 [41]; and CD44, CD79, CD90, CD105 [39]. They are usually negative for hematopoietic markers, such as CD34 e CD45 [39].

3.1. Synovial membrane-derived MSCs

MSCs from different sources present unlike characteristics, such as phenotype, proliferation capacity, and differentiation ability [29, 38], thus affecting the cell biological properties and therapeutic potential [4]. These intrinsic differences are influenced by the tissue of origin environmental factors [12, 29]. There has been an increasing interest in developing cell-based therapies, with the use of MSCs, with or without scaffolds. However promising for regeneration therapies, the most suitable source of MSCs for cartilage and for muscle repair still remains controversial [4].

Bone marrow-derived MSCs (BM-MSCs) were the first stem cells applied in cartilage injury therapy studies. Although their chondrogenic potential has been established, the improper differentiation of BM-MSCs during cartilage regeneration has been repeatedly reported [4], leading to the need to study alternative MSC sources.

3.1.1. Characterization of synovia-derived MSCs

Synovia membrane-derived MSCs (SM-MSCs) were first identified in 2001 [31] and are a promising source of MSCs regarding musculoskeletal therapies, due to their intrinsic characteristics. They present a high self-renewal ability [31, 40], superior potential for chondrogenic differentiation [3, 4, 10, 12, 30, 32, 37], and a high proliferative capacity [3, 4, 10, 12, 29, 32, 37, 40], both compared to nonjoint (bone marrow, adipose tissue, and umbilical cord) and joint tissues (AC and synovial fluid) [4]. SM-MSCs have been successfully differentiated into osteogenesis, chondrogenesis, and adipogenesis [31, 35], as well as toward myogenesis, but at a minor extent. They maintain their intrinsic differentiation characteristics, regardless of the donor, age [4, 6, 31], cell passage number, or cryopreservation [31]. SM-MSCs can produce hyaline-like cartilage tissue, under specific conditions, becoming a promising approach to cartilage injury therapies [4].

SM-MSCs are easily expandable in vitro, maintaining a stable profile, and retaining their multidifferentiation ability, even over 10 passages [4, 12, 31, 32]. The SM is easy to harvest [40] and can be collected from any joint, without impairing the AC tissue [3]. It can be obtained arthroscopically, with a minor degree of invasiveness [10] through a small biopsy [31] and with minor complications at the donor site [12]. It is a quite accessible source, as the SM is routinely removed from OA patients for knee replacement or other arthroscopic interventions [15]. It can be cryopreserved and stored for future use, as it is not negatively influenced by cryopreservation methods [31].

3.1.2. The synovial membrane as a niche to SM-MSCs

The SM is composed of two different layers: the synovial lining, rich in fibroblast-like synoviocytes and macrophage-like synoviocytes, and the subintimal layer, constituted by fibrous tissue, blood vessels, and immune cells. The origin of SM-MSCs has been speculated to be from the synovial lining, as they are biologically similar to the fibroblast-like synoviocytes [4]. SM-MSCs are more closely related, developmentally, to chondrocytes, in comparison to other MSC sources [17] revealing proximity of the gene expression profiles of SM-MSCs and chondrocytes when compared, for example, to BM-MSCs, adipose tissue-MSCs and umbilical cord-MSCs [4, 42], which further support the SM-MSCs' superiority in chondrogenesis differentiation. SM-MSCs have been found in healthy joints, confined to the subintimal layer, but also in OA individuals, in a more diffuse distribution through the joint tissues [4]. As there has not yet been established a specific marker to identify MSCs, it is not possible to address the topographic distributions of MSCs in the joint [31, 43]. Various theories have been proposed for their origin. They can be derived from precursor cells to enter the joint from the circulation and/or they can have been recruited from the BM through vascular channels [31].

The SM is a very responsive tissue upon cartilage injury. It responds to full thickness defects with proliferation and chondrogenic differentiation [44], but the most common source of reparative cells in these cases are the BM-MSCs, as they can infiltrate directly from the subchondral part of the defect into the joint space [4]. In partial defects, a positive response from the SM can be observed, by the formation of a continuous layer of SM-MSCs extending from the SM across the normal AC into the injury site [45]. The recruited SM-MSCs migrate toward the lesion site under chemotactic signals and undergo chondrogenesis, stimulated by the transforming growth factor β (TGF-β) [46].

The synovial fluid (SF) is a viscous and clear, rich in hyaluronic acid, liquid in intimate contact with the AC and the SM. It represents a route for exogenous cells to access the cartilage injury site [4] and 'free-floating' MSCs have been identified and isolated. Different theories have been proposed to address the origin of the MSCs present in the SF. They can have their origin in the disrupted cartilage, bone, SM, periosteum, and also in the BM itself. Regarding the SM, cells can shed into the lumen or through reported vascular pericytes of the SM [43].

3.1.3. Harvesting and isolation methods for SM-MSCs

For regenerative medicine purposes, it is helpful to harvest the greatest cell number, with greatest potential, from the smallest amount of tissue possible, minimizing the harvesting impact on

the tissue source. Isolation methods have not been exhaustively characterized. For example, Sugita et al. proposed an isolation method without filtration to be more effective in collecting more cells from a smaller sample [37], in contrast to the common ones, which undergo filtration.

SM-MSCs harvested from OA joints have the same osteogenic and chondrogenic differentiation potential [41, 47], although they present superior proliferation abilities, in comparison to SM-MSCs from healthy joints [47].

3.1.4. SM-MSCs in vitro culture

Fickert et al. reported that after the harvested cells adhered in monolayer cultivation, the subtype of cells expressing CD markers enriched remarkably [41]. During *in vitro* culture, SM-MSCs become homogeneous populations after the first passages. During expansion, hematopoietic and endothelial markers almost disappear and they present a stable molecular profile between passage 3 and 10 [31].

Enriched subpopulations of SM-MSCs present more efficient chondrogenic differentiation abilities [4]. A possibility of cell selection to get more homogeneous populations [40] involves the use of growth factors.

Ashton et al. first reported chondrogenesis of MSCs [48]. A specific medium for *in vitro* chondrogenesis was described containing transforming growth factor beta (TGF-β), dexamethasone [49], and bone morphogenetic proteins [50]. Evaluation of MSCs' chondrogenesis potential is currently performed with a micromass technique [21].

Another group suggested the combination of growth factors: insulin-like growth factor I (IGF-I), basic fibroblast growth factor (FGF), and TGF-β_1, applied early in culture, with a posterior addition of TGF-β_1, and reported an enhanced proliferation of SM-MSCs with chondrogenic potential. It has been well established that growth factors induce important effects on MSCs' differentiation potential. TGF-β is known to promote collagen II and proteoglycan expression; furthermore, it enhances cell recruitment into the prearticular tissue, regulating MSCs' condensation during cartilage formation; TGF is also involved in the expression of Sox-9, a gene responsible for a major regulation of the chondrogenesis differentiation. FGF promotes proteoglycan synthesis and IGF-I plays a role in chondrogenesis regulation, augmenting the expression of chondrogenic markers, thus, impacting on skeletal growth [51].

Although there has been some concern about the chondrogenic stability of SM-MSCs *in vitro* [52], a recent study reported SM-MSCs to present a significantly higher expression of chondrogenic markers and a stable chondrogenic phenotype [47].

3.1.5. SM-MSCs' therapeutic applications

Therapeutic strategies with resource to SM-MSCs have been developed in the past years, mostly for osteoarticular tissue regeneration. **Table 1** summarizes the most relevant studies applying SM-MSCs in *in vivo* models, which will be extensively addressed in this section.

Intra-articular (IA) administration of SM-MSCs has been widely reported. Nakamura et al. reported intra-articular transplantation of SM-MSCs in a pig model, inducing repair of

SM-MSC donor species	Lesion model		Delivery mode	Refs.
	Host	Lesion type/site		
Rat SM-MSCs	Rat	Patellar groove osteochondral defect	Intra-articular magnet	[3]
Porcine SM-MSCs	Pig	Femoral condyle full-thickness osteochondral defect	IA administration	[30]
		Chondral defect in the knee femoral condyle	Scaffold-free TEC	[6, 8, 33, 58]
Rabbit SM-MSCs	Rabbit	Femoral trochlear groove full-thickness osteochondral defect	Local adherent technique	[5]
			Cell aggregates	[54]
			Collagen gel + periosteum coverage	[40]
		Femoral trochlear groove osteochondral defect	PRP gel	[10]
		Femoral trochlear groove partial-thickness osteochondral defect	IA administration after chondroitinase ABC treatment	[53]
		Femoral condyle full-thickness osteochondral defect	Tissue construct: fibrin glue + polyglycolic acid netting	[59]
Human SM-MSCs	Rabbit	Femoral trochlear groove full-thickness osteochondral defect	Cell aggregates	[54]
	Mouse	Tibialis anterior muscle injury	Injection	[22]

Table 1. Animal studies applying SM-MSCs for musculoskeletal repair.

cartilage defects. Cell adherence to the injury site was observed through fluorescent labeling [30]. Recently, SM-MSCs from OA individuals have been reported to suppress T-cell proliferation and to suppress T-reg populations *in vitro*, when cocultured with allogenic lymphocytes. Thus, indicating their ability to suppress the immune response and prevent OA development [15, 35]. Yan et al. reported SM-MSCs' ability to prevent autoimmune disease and recover self-tolerance after repeated IA administrations of SM-MSCs from OA individuals to a collagen-induced arthritis murine model. They observed a superior histological and clinical scores in treated individuals, with inferior tumor necrosis factor α (TNF-α), interferon gamma (IFN-γ), and interleukin (IL) 17A, and increased IL-10 levels [15]. Another group also reported their ability to display indoleamine 2,3-dioxygenase (IDO) activity, after stimulation with IFN-γ and/or TNF-α that has been recently correlated to the T-cell suppressive mechanism in humans [35].

To address the problem of dispersion of injected cells inside the articular joint capsule, Hori et al. proposed the use of an intra-articular magnet, to conduct the IA administered cells to the injured site, where an intra-articular magnet is placed. They successfully reported the mobilization of the injected cells to the lesion site [3].

Nevertheless, IA administration has also been reported to be insufficient, as it results in an increased number of T-cell recruitment, relating to the development of synovitis [53]. A possible explanation for this reaction, in comparison to other therapeutic techniques, can be the

number of SM-MSCs injected to the joint, which is considerably higher when performing IA injection and that most of them adhere to surrounding tissues [5].

Koga et al. compared the effectiveness of IA injection of SM-MSCs with a local adherent technique using a rabbit model. They reported an *ex-vivo* 60% attachment to the defect in the local adherent technique, 10 min after the beginning of the procedure. *In vivo*, they registered a 24 h attachment that showed improved histological scores for 24 weeks, in comparison to the IA administration technique. This technique was scaffold free, with no periosteal coverage of the inserted cells. They also performed the *ex-vivo* technique in humans, with similar results [5].

Another study applied SM-MSCs in a full-thickness defect, with collagen gel covered with periosteum, in a rabbit model. They showed that SM-MSCs undergo differentiation and evolve into chondrocytes, responding to environmental cues, and remain active for at least 24 weeks. They also demonstrated an abundant cartilage matrix production. However, the cartilage became thinner after the 24 weeks, suggesting long-term incomplete healing process [40].

Later, the same group investigated the possibility to transplant aggregations of SM-MSCs for cartilage regeneration. The aggregates were produced easily by the hanging drop technique. They reported an improved cartilage matrix synthesis from SM-MSC aggregates, compared to SM-MSCs cultured in monolayer. They adhere to the defect by surface tension. Successful cartilage repair was achieved with transplantation of a low-density aggregate. These findings suggest a way to improve cartilage repair techniques, with minor loss of SM-MSCs. However, they propose the use of fibrin glue to improve results in a future study [54].

Lee et al. reported the application of SM-MSCs into the cartilage defect of rabbits, embedded in platelet-rich plasma gel (PRP), as it was previously studied with chondrocytes. PRP is defined as a volume of plasma fraction of autologous blood that is composed of a higher platelet concentration. It is described to be an important source of growth factors, enhancing chondrogenesis and proliferation of MSCs. They concluded that SM-MSCs in association with PRP showed improved results, in comparison to PRP alone. However, the applicability of this technique may not be suited for all osteochondral defects, and the clinical benefits of PRP are still controversial [10]. Chiang et al. also applied a PRP hydrogel with SF-MSCs in a porcine model, with satisfactory results [55].

Lee and his group also proposed a treatment with chondroitinase ABC in a rabbit *ex-vivo* partial defect model, to promote the adhesion of transplanted SM-MSCs. The proteoglycan antiadherent properties impact on the cell adhesion ability, to the cartilage surface [4, 53]. Chondroitinase ABC is an enzyme that depolymerizes the glycosaminoglycan side chain, thus, exposing to the underlying fibronectin, which presents cell adhesive properties. As such, this approach enhanced cell adhesion. However, the repaired tissue showed lack of hyaline-like cartilage content [53].

It is generally accepted that a three-dimensional (3D) environment enhances cell proliferation and differentiation abilities. Artificial scaffolds, composed of synthetic polymers or biomaterials, are often used. However, they are related to various issues with regard to long-term safety, such as degradation *in situ*, retention, and transmission of infectious agents. Scaffold-free tissue-engineered constructs (TECs) were developed in order to overcome the previous

drawbacks. MSCs are cultured in monolayer with addition of ascorbic acid and are then submitted to shear stress, resulting in their detachment and spontaneous contraction to form the 3D structure, similar to what is observed with collagen gels [56].

TECs based on allogenic SM-MSCs have been applied on cartilage defects from varied species. Shimomura et al. used a TEC based on SM-MSCs derived from immature and mature pigs, in order to address the age dependency in chondrogenic and proliferation abilities of SM-MSCs. No differences were reported between the groups, suggesting no age dependency [6]. Ando et al. similarly used a porcine allograft model, with a basic TEC composed of collagen I, III, vitronectin, and fibronectin. The TEC showed to have stable adhesion to a porcine cartilage matrix, in an explant culture, possibly due to the adhesion properties of fibronectin. When cultured in chondrogenic medium, enhanced expression of glycosaminoglycans and chondrogenic matrix genes, as collagen II and aggrecan, was observed [33, 56], suggesting that SM-MSCs in the TEC retain their chondrogenic potential [56]. They also proposed a xeno-free system for the development of this technique, as the TEC is produced without an exogenous scaffold, with autologous serum and MSCs. A chondrogenic-like tissue was formed in the defect, *in vivo*, with similar mechanical properties to a normal cartilage and progression of OA phenomena was prevented, compared to untreated defects [33].

In terms of human SM-MSCs, investigators proved human SM-MSC-derived TECs to be rich in fibronectin and vitronectin. This group demonstrated cells' ability to adhere to human chondral fragments [56], as it was previously demonstrated in the pig model. They also applied a xeno-free technique, by using human serum [5, 56]. Autologous human serum is reported to be more effective in promoting SM-MSCs' proliferation, in comparison to other MSC sources [5, 10, 57].

Later, Fujie et al. developed a similar TEC in a porcine model and reported a mechanical vulnerability at the repaired tissue boundary, indicating commitment of long-term durability from the repaired tissue, regardless of the apparent secure tissue continuity and histological quality [8]. Ando et al. developed a new TEC in 2012, showing to have histological defects at the superficial layer of repaired cartilage that presented a stiffness surface and lower water-retaining capacity. Thus, improvement is still needed regarding TEC strategies for cartilage defects' long-term repair [58].

Pei et al. reported the use of an allogenic SM-MSC-based premature tissue construct in a full-thickness osteochondral defect. They combined SM-MSCs from a rabbit with fibrin glue and seeded into polyglycolic acid netting. Incubation in a bioreactor lasted 1 month, with growth factor enrichments. After 6 months, the defects were covered by a hyaline-like tissue, well adhered to the surrounding healthy cartilage, presenting collagen II and glycosaminoglycans. However, contamination with macrophages was an issue in the *in vitro* assays [59].

A recent report was the first to investigate OA therapies resorting to exosomes, which result in the paracrine secretion of trophic factors by MSCs. They compared the therapeutic abilities of SM-MSC exosomes and the ones produced from human induced pluripotent stem cells in OA, using a mouse model. Stronger effects were observed by human induced pluripotent stem cell exosomes, representing a possible alternative to MSCs for OA treatment [17].

Regarding human *in vivo* research, Sekiya et al. reported a promising study involving 10 individuals with articular defects. SM-MSCs were successfully applied locally and rested for 10 min for adherence, as the same investigators reported before in pigs and rabbits. The therapy efficacy *in vivo* was evaluated, according to MRI, histological, and clinical scores. Only one patient presented fibrous cartilage in the deep-zone, although, in general, the results were satisfactory and promising [60].

De Bari and his group investigated the potential use of SM-MSCs for muscle repair in mdx mouse model for DMD. They demonstrated that human SM-MSCs had the capacity to contribute for the formation of myofibers and long-term persisting SC. The cells were injected into the blood stream, engrafting in several tissues. However, they only acquired muscle phenotype within the skeletal muscle tissue, verifying their sensitiveness to environmental cues. They observed that the administration of SM-MSCs restored the sacrolemmal expression of dystrophin and rescued the expression of mouse mechano-growth factor (MGF). MGF is involved in muscle repair and maintenance but is undetectable in dystrophic mdx mouse, even after mechanical stimulation. They also reported that a subpopulation of the injected cell remained for several months as SC. These findings suggest the significant role of SM-MSCs in restoring pathophysiologic features of the dystrophic muscle in the animal model [22].

4. Conclusion

In summary, MSCs play an important role in embryonic development, postnatal growth, repair, and regeneration mechanisms, as well as in maintaining tissue homeostasis, and synovial membrane mesenchymal stem cells are a promising, easily available source. Despite relevant recent advances, challenges still remain on the use of MSCs as standard therapeutic options for clinical applications.

Although BM-MSCs remain the most studied source of MSCs, as they were the first to be characterized, SM-MSCs are an easily available source with proven enhanced chondrogenic, osteogenic, and myogenic differentiation ability. Nonetheless, their characterization, as established by the expression of specific cell surface markers, may be affected by interindividual heterogeneity and major differences in cellular marker expression profiles may be found in nonhuman species.

The most effective administration route for SM-MSC application *in vivo* remains to be defined and the genetic stability of the cells must be assured both *in vitro* and *in vivo*. Also, the biomechanics and secretory profile of these cells must be further studied, in order to comprehend the mechanism of regenerative capacity of these cells and secretion profile in signaling factors, growth factors, cytokines, and other bioactive molecules, and their role on chondrogenic as well as osteogenic and myogenic differentiation.

SM-MSCs present themselves as a promising source of MSCs that are becoming the targets of several research groups worldwide. Their application *in vivo* in preclinical and clinical trials is envisioned for the therapeutics of musculoskeletal disorders.

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Conflict of interest

The authors declare no conflicts of interest.

Abbreviations

AC	articular cartilage
ACI	autologous chondrocyte transplantation
CD	cluster of differentiation
CFU-F	colony-forming unit fibroblast
BM	bone marrow
DMD	Duchenne muscular dystrophy
FGF	fibroblast growth factor
HLA	human lymphocyte antigen
IA	intra-articular
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon gamma

IGF-I	insulin-like growth factor I
IL	interleukin
ISCT	International Society of Cellular Therapy
MGF	mechano-growth factor
MRF	myogenic regulatory factors
MSCs	mesenchymal stem cells
OA	osteoarthritis
PRP	platelet-rich plasma gel
SC	satellite cells
SF	synovial fluid
SM	synovial membrane
TEC	tissue-engineered construct
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor α

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Chapter 7

hiPSC-Based Tissue Organoid Regeneration

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Additional information is available at the end of the chapter

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Abstract

Induced pluripotent stem cells (iPSCs) are generated from terminally differentiated cells and have the potential to differentiate to any organs originated from the embryonic germ layers. Extensive effort has been made to establish protocols for direct *in vitro* conversion of human iPSCs (hiPSCs) to different cell types/organs. Importantly, hiPSCs can be generated from patients with known genetic mutations that predispose to high-risks of specific disease development. Thus, the hiPSCs technology provides unlimited resources for creating patient-specific disease models. hiPSC-derived three-dimensional "organoid" models have recently emerged as a powerful tool to recapitulate the physiologically-relevant process of disease progression *in vitro*. In this chapter, we will discuss the current advancement of organoid regeneration from hiPSCs and the applications of hiPSCs-derived organoids. The limitations and challenges of this approach will also be discussed here.

Keywords: disease modeling, induced pluripotent stem cells, organoid, organ-on-chip, tissue regeneration

1. Introduction

Induced pluripotent stem cells (iPSCs), generated directly from terminally differentiated cells [1], can differentiate toward all three embryonic germ layers - ectoderm, mesoderm, and endoderm. iPSCs can give rise to diverse cell types such as neurons, cardiomyocytes, and hepatocytes under defined conditions [2–4], and thus may provide a useful tool for studying human organ development. Human iPSCs (hiPSCs) also open new avenues for patient-specific or personalized disease modeling and therapies [5]. In the following sections, we will summarize the current advances in hiPSC-derived organoid differentiation and discuss the applications of these hiPSC-derived organoids in pre-clinical and clinical areas.

2. Generation of hiPSC

2.1. Protocols for generating hiPSCs

Dr. Yamanaka first reported the generation of hiPSCs from fibroblasts using four transcriptional factors (POU5F1, SOX2, KLF4, and MYC) [6]. There are many protocols to further improve the original method. The first improvement was to minimizing the integration risks such as using non-integrating adenoviral vectors, transfection of mRNA, and using cell-penetrating peptide-tagged reprogramming factors [7]. Transgene-free hiPSC generation protocols have been published by multiple groups [8]. Using small molecules such as valproic acid, sodium butyrate, PD0325901, and others to create iPSCs has been reported [9–11]. Haase et al. reported a new non-transgenic protocol to generate hiPSCs from patient cord blood CD34+ cells using CytoTune™ Sendai reprogramming vectors under the exclusive usage of animal-derived component-free (ADCF) materials and components [12]. Recently, non-integrative and non-viral mRNA reprogramming technology has been reported for hiPSC generation [13]. Rapid, efficient, and safe strategies which are compliant with standard Good Manufacturing Practice (GMP) regulations pave the way for hiPSC clinical applications.

2.2. Genome editing of hiPSCs

Genome editing in hiPSCs provides a valuable tool for disease modeling, mechanism study, and gene therapy. A line of technology utilizing engineered nucleases consisting of sequence-specific DNA-binding domains attached to a non-specific DNA nuclease have been developed. These cutting-edge technologies allow researchers to manipulate entire genomes, including specific genes, intergenic regions, promoters, enhancers, silencers, and insulators. After zinc finger nucleases (ZFNs, first-generation) and transcription activator-like effector nucleases (TALENs, second-generation), the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) technology is the third-generation editing tool. Despite the difference in the nucleases, the common mechanisms involve inducing DNA double-strand breaks (DSBs) in targeted DNA. Compared to TALEN and ZFN, CRISPR/Cas9 has become the system of choice because of its features such as high feasibility, high affordability, and precise targeting.

3. hiPSC-based tissue regeneration

hiPSC-derived organoids are valuable resources and tools for disease modeling, organ development research, and therapy screening. The current established hiPSC-derived organoids are listed in **Table 1** (adapted from Shi et al. [1]).

3.1. Ectoderm-derived tissues

Ectoderm is one of the three germ layers and the most exterior layer in the human embryo. It covers the outside of the embryo. The ectoderm gives rise to the central nervous system

Organoids	Applications	Refs.
iPSC derived organoid model		
Brain organoids	Modeling autism disorder	[14]
	Modeling ALS disease	[15]
	Modeling Parkinson's disease	[16]
	Modeling Zika virus infection	[17]
	Modeling Seckel syndrome	[18]
Brain-region specific organoids	Modeling Zika virus infection and human brain development disease	[19]
Breast organoid	Breast cancer research	[20]
Cystic organoids	Modeling Alagille syndrome, polycyctic liver disease and cystic fibrosis	[21]
Fallopian tube organoids	Ovarian cancer research	[22]
Liver bud	Organ-bud transplantation for regenerative medicine	[23]
Lung organoids	Lung development and lung disease modeling	[22, 25]
Pancreas	Pancreatic disease model	[26]
Retinal organoids	Modeling glaucoma	[27]

Table 1. Summary of hiPSC- and ESC-derived organoids, adapted from Shi et al. [1].

(the brain and spinal cord), the peripheral nervous system, the sensory epithelia of the eye, ear, and nose, the epidermis and its appendages (the nails and hair), the mammary glands, the hypophysis, the subcutaneous glands, and the enamel of the teeth (**Figure 1**).

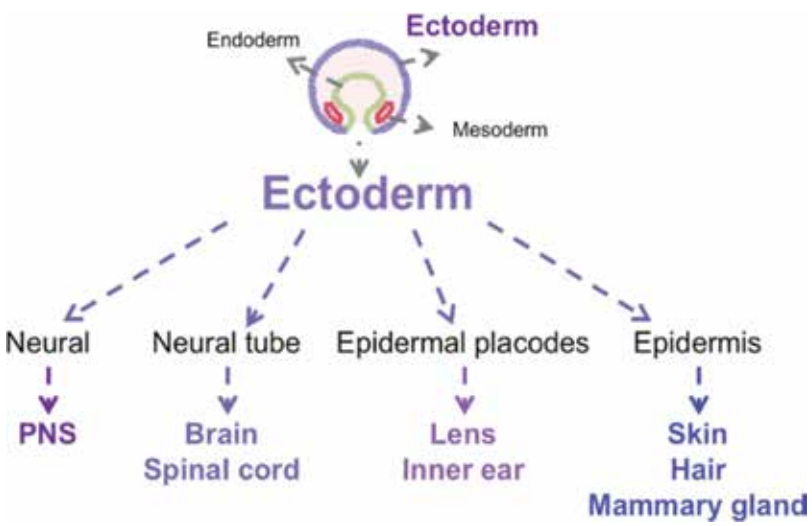

Figure 1. Summary of the organs originated from ectoderm.

3.1.1. Neuronal tissue regeneration

Several protocols have been developed for the *de novo* differentiation of hiPSC into cell types comprising the central nervous system (**Figure 2**). In general, protocols utilize either a monolayer culture condition, in which the neuroectoderm is further pushed toward spinal or cortical fates via neural rosette formation, or a three-dimensional culture system leading to the formation of neural organoids that again possess features of either cortical or spinal cell types. Here, we discuss the critical components of cortical and spinal organoid differentiation protocols.

Successful formation of a cortical organoid depends upon the appropriate temporal- and regional-specific expression of several proteins and transcripts. The first method of this differentiation was presented by Lancaster et al. [28] in which the group relied upon intrinsic

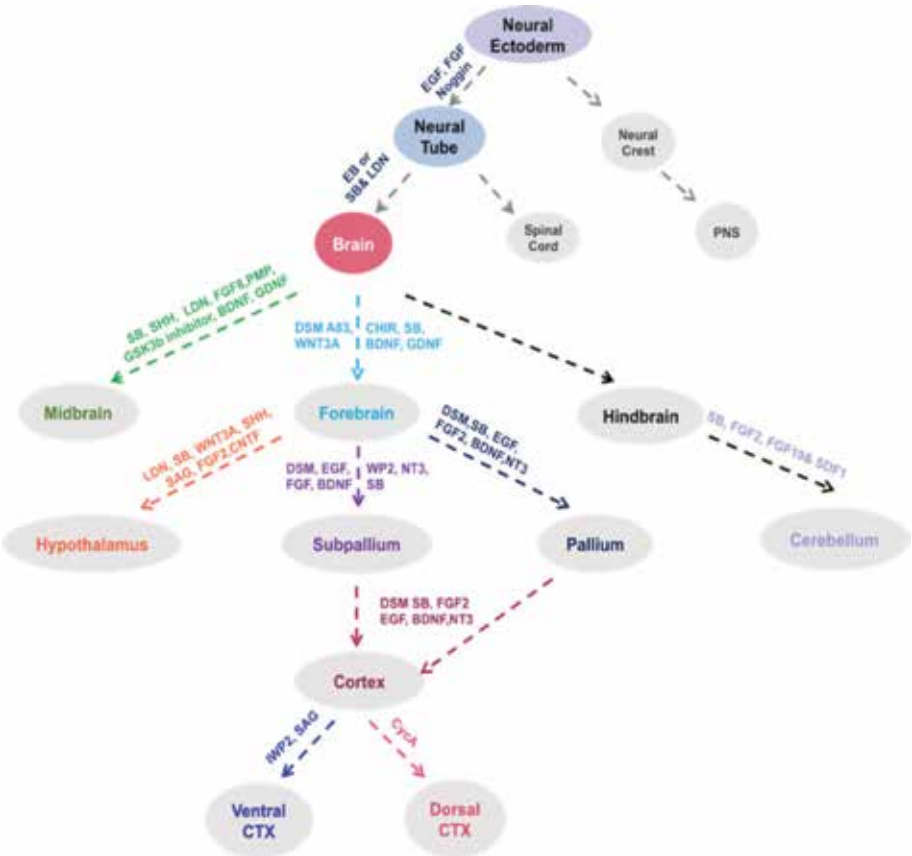

Figure 2. Specification of iPSC-derived neural tissue and exogenous factors used for derivation of organoids from iPS cells. Arrows indicate the temporal flow of tissue lineage as described by human development. Colored text denotes the small molecules that have been used to derive organoid models of each tissue (in gray) from iPSC spheroid or EB culture. Brain regions: CTX-Cortex and PNS: Peripheral nervous system. Growth factor and small molecules: BDNF, Brain-Derived Neurotrophic Factor; CNTF, Ciliary Neurotrophic Factor; CycA, Cyclophilin A; DSM, Dorsomorphin; EGF, Epidermal Growth Factor; FGF, Fibroblast Growth Factor; GDNF, Glial Cell-Derived Neurotrophic Factor; IWP2, WNT inhibitor; LDN, LDN-193189; NT3, Neurotrophin-3; SAG, Smoothened Agonist; SB, SB431542; SDF, Stromal cell-derived factor; SHH, Sonic Hedgehog; PMP, Purmorphamine (Adapted from Lullo and Krigstein [29]).

self-organization, a droplet of gelatinous matrix, and spinning bioreactors to drive neural-specific embryoid bodies toward developing cerebral structures.

Based on this initial protocol, several groups have published tangential methods that have improved the cortical organoid model. Pasca et al. [30] developed a differentiation method resulting in the generation of exclusively excitatory neurons reminiscent of the dorsal telencephalon, as well as the derivation of non-reactive astrocyte-like cells. Dual SMAD inhibition has been used to induce neural-ectoderm differentiation in suspension, which results in high-efficiency temporal and spatial organization of forebrain organoids [19, 30, 31]. Groups have also implemented transcriptional profiling to investigate the cell type composition of the mature organoid and to compare the overall maturity to that of the developing human brain [19, 30].

One of the most critical features for defining or characterizing a neuron from iPSCs is the neuron's function and physiology. Many groups have assayed the physiology of the developed organoids, implementing calcium imaging or patch-clamp physiology to describe spontaneous activity and expression of specific neurotransmitter receptors [19, 28, 30]. With the addition of an exogenous matrix built of hydrogel, another model of cerebral organoids could reach a larger size over a shorter duration in culture and express the oligodendroglial-like marker Olig2 [32].

In addition to modeling the brain and its specific regions, neural organoids have also been developed for modeling the immature spinal cord and motor nerve units [33, 34]. Experimentation with different extracellular matrix components and rigidity led to the dorsal-ventral patterning of neural cysts within nine days of culture [33], resulting in the immature modeling of the human spinal cord. Another group described a method for generating motor nerve organoids that developed a polarized axon fascicle [34]. Although the above protocols have their limitations, it is evident that organoid technology is rapidly moving toward the goal of forming nervous systems.

3.1.2. Non-neural tissue regeneration

3.1.2.1. Skin

Skin is the largest organ of the body and provides a barrier to protect the interior from the external environment. Human skin is the first barrier system that is vital for homeostasis. Protocols using hiPSCs to generate human skin or skin components have been developed. Regeneration of human skin typically composed of 2D and 3D methods. Keratinocytes are major epithelial components in the skin. Researchers have developed protocols using defined medium and chemical/cytokines generate keratinocyte them. Itoh et al. used a combination of retinoic acid and bone morphogenetic protein (BMP) 4 to induce ectoderm epithelial cell differentiation from the patient-derived hiPSCs [35]. The same group later generated hiPSC-derived dermal fibroblasts, together with keratinocytes, to build 3D skin equivalents using an air-liquid interface culture [36]. The skin contains not only keratinocytes and fibroblasts, but also other skin appendages (eg. sweat glands, sebaceous glands, and hair follicle) and cells from different germ layer origins (fat cells, neurons, immune cells, muscles, blood vessels, and melanocytes. So far, the *in vitro* differentiation protocols have only been successful in developing dermal fibroblasts, keratinocytes, and melanocytes [37, 38]. Creating a full layer of skin tissue is still unfulfilled (**Figure 3**).

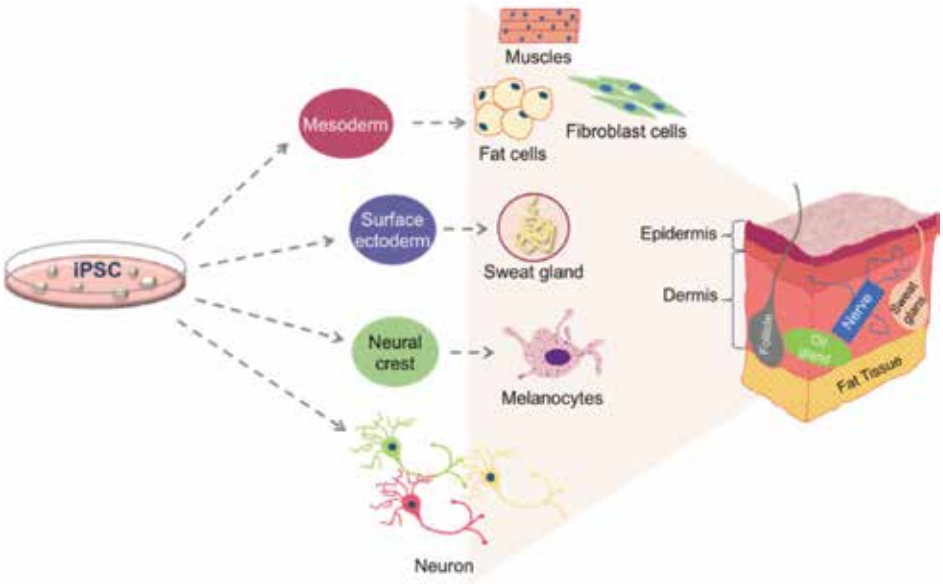

Figure 3. The hiPSC-based regeneration strategy for a full-layered human skin.

3.1.2.2. Mammary gland

Mammary epithelial cells originate from non-neural/surface ectoderm cells, which co-exist with neural ectoderm cells at the same embryonic stage. Although mouse mammary gland development has been well studied, the human breast development is still poorly understood due to numerous differences between the mammary glands of the two species. In addition, questions regarding human mammary stem cell identity, mammary epithelial differentiation hierarchy, and the effects of ovarian hormones on mammary development are major obstacles for *in vitro* mammary gland regeneration.

Taking a cue from the understanding of human embryonic mammary gland development [39, 40], Qu et al. conceptualized that the first step for *in vitro* induction of mammary differentiation from hiPSCs was to pattern hiPSCs in to non-neural ectoderm, thus enriching mammary progenitors. The group developed a reliable two-step protocol to generate human mammary-like organoids from hiPSCs [20]. These organoids express luminal, basal, and breast-specific markers. Despite these novel findings, this *in vitro* system needs to be improved to fully recapitulate the formation of mammary ductal and alveolar structures.

3.2. Mesoderm-derived tissues

The mesoderm is formed through a process called gastrulation around the third week of embryonic development. Initially, mesoderm is segmented into three crucial compartments; the paraxial mesoderm (PM), the intermediate mesoderm (IM), and the lateral plate mesoderm (LPM) (**Figure 4**). The PM, also known as presomitic or somitic mesoderm, gives rise to embryonic structures of the sclerotome, myotome, and dermomyotome, which later develop into many

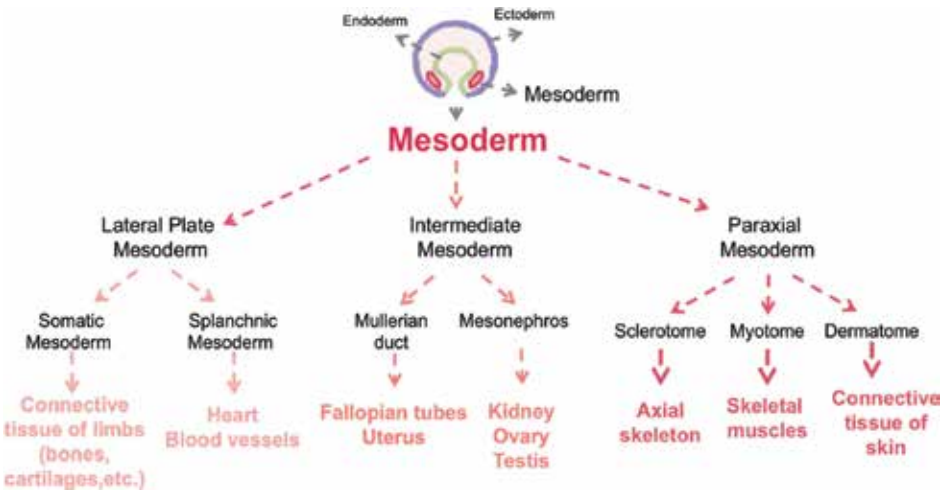

Figure 4. Mesoderm subdivision and mesoderm-originated tissue development.

adult tissues, including most of the axial skeleton, skeletal muscles, and connective tissues of the skin. The IM, which lies between the PM and LPM, differentiates into the urogenital duct system and gives rise to the kidneys, gonads, Wolffian (male) or Müllerian (female) ducts, fallopian tube, uterus, and the adrenal glands. The LPM is located on the side of the IM and is split horizontally into two layers: splanchnic mesoderm and somatic mesoderm. These layers contribute to the formation of the heart, blood vessels, and blood cells as well as to the connective tissue of the limbs and the space between these layers develop into the body cavity. This section summarizes the current hiPSC-derived 3D organoid differentiation research for tissues of mesodermal origin.

3.2.1. Kidney

Adult humans have a limited number of nephrons which do not increase during life but rather decay with age through attrition or disease. Currently, there is no known treatment accessible for nephron renewal in patients with chronic or end-stage kidney disease. Recent hiPSC-based tissue regeneration studies have provided the novel sources for nephron progenitor cell (NPC) production and potentially kidney regeneration. This section summarizes the current protocols to generate and maintain NPCs and 3D human kidney organoids.

The kidney is derived from IM which forms a urogenital ridge on either side of the aorta. Current multistep-directed differentiation methods are intended to recapitulate these crucial stages of renal embryonic development. Studies for the critical transcriptional regulation process and signaling pathways contribute to the better understanding of each stage of renal development (**Figure 5**). Importantly, these studies enable us to recognize the factors that direct cell fate decisions and have been the basis for establishing the current differentiation protocols and culture conditions [41, 42].

hiPSC-derived 3D human kidney organoids that led to the generation of more complex and partially self-organizing organoids [43–46] have been established (**Figure 6**). Takasato et al. [43, 44]

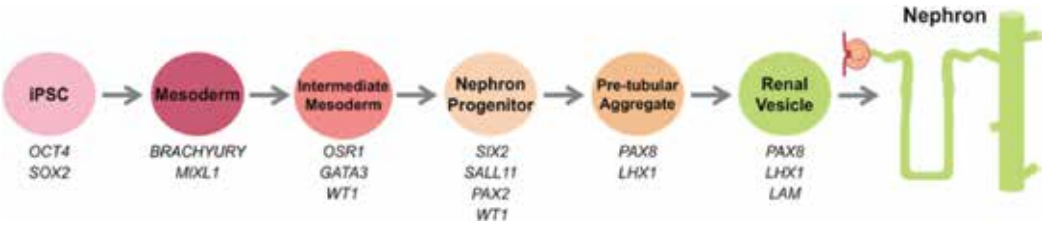

Figure 5. Schematic representation of the differentiation stages into NPCs and kidney organoids and markers that identify each stage. GATA3, GATA Binding Protein 3; LAM, laminin; LHX1, LIM homeobox 1; LPM, lateral plate mesoderm; MIXL, Mix Paired-Like Homeobox; OCT4, POU class 5 homeobox1; OSR1, odd-skipped related transcription factor 1; PAX2, paired box 2; PAX8, paired box 8; SALL1, spalt-like transcription factor 1; SIX2, SIX homeobox 2; SOX2, SRY-box 2;WT1, Wilms tumor 1.

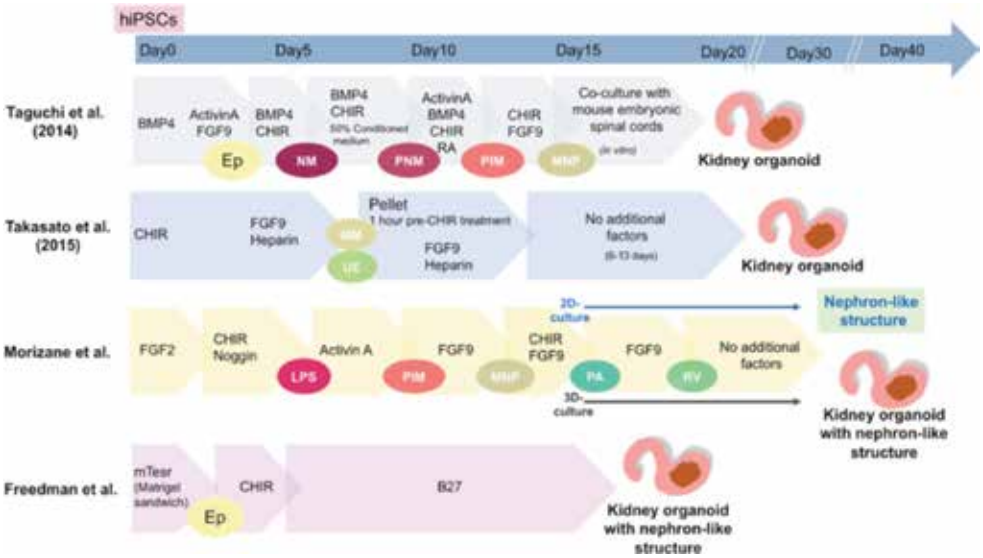

Figure 6. Protocols for the directed differentiation of hiPSC into kidney organoid. The timescale is shown at the top (shortened after 20 days) and Day 0 marks the hiPSC stage. Oval images represent the obtained cell types-like in each stage, namely: Ep, epiblast; LPS, late primitive streak; NM, nascent mesoderm; PNM, posterior nascent mesoderm; PIM, posterior intermediate mesoderm; UE, ureteric epithelium; PA, pre-tubular aggregate; RV, renal vesicle. Growth factors and small molecules; BMP4, Bone Morphogenetic Protein bone 4; CHIR, CHIR99021; FGF, Fibroblast Growth Factor; RA, Retinoic Acid; VitD3, vitamin D3; WNT3A, Wingless Type Family member 3A; mTeSR1, defined iPSC medium and B27-Serum free cell culture supplements. Figure adapted from (Jacqueline Kai et al. [47]).

reported the generation of self-organizing kidney organoids using CHIR99021 and fibroblast growth factor (FGF) 9/heparin in a monolayer culture followed by cell pelleting. The transcriptional profiling of resulting organoids exhibits significant similarity to the first-trimester embryonic human kidney.

On the other hand, Morizane et al. [45] have patterned mesoderm into PM, IM, and LPMs and generated NPCs by mediating graded signals of GSK-3β inhibitor CHIR99021, Noggin, and Activin A. In this study, renal vesicle was formed by transiently treating the NPCs with the CHIR99021 and FGF9, following self-organizing differentiation into podocytes, proximal tubules, loop of Henle, and distal tubules in both 2D and 3D culture. Alternatively, a shorter

and more straightforward protocol has been developed by the group of Bonventre and collaborators. Bonventre group *et al.* demonstrated the formation of nephron-like structures as well as endothelial-like cells that were arranged into cords and expressed the endothelial markers CD31 and von Willebrand factor [46].

3.2.2. Cardiomyocytes

Cardiovascular disease remains the leading cause of death worldwide. It encompasses an extensive range of clinical conditions due to genetics, physiologic and metabolic circumstances as well as drug toxicity. Most heart diseases are associated with severe damage to, or loss of, cardiomyocytes (CMs), and mammalian CMs have a limited regenerative capacity [48]. The recent advancements in the field of hiPSC-derived CMs (hiPSC-CM) offer unique opportunities for not only disease modeling and personalized drug efficacy/toxicity screening but also for stem cell-based cardiac regenerative therapy [49–51].

CMs arise from mesoderm, which is further specified into cardiac mesoderm and cardiac progenitor cells by three families of extracellular signaling molecules: WNT, FGF and TGFβ superfamily ligands (WNT3a, bone BMP4, Nodal and Activin A). The expression of these ligands in a spatiotemporal manner defines the mesodermal cell fate and prime CM differentiation [52, 53]. Several groups successfully mimic these signaling processes *in vitro* to generate hiPSC-CM, which is summarized in **Table 2** (adapted from Smith et al. [54] and Burridge et al. [55]).

Method	Culture condition	Differentiation format	Mesoderm induction	Cardiac specification factors	Cardiac Differentiation factors	Ref.
Suspension EB in StemPro34	Knock-out serum Replacement (KSR)/FGF2	StemPRo 34	Activin A, BMP4	VEGFA, DKK1	VEGFA, FGF2	[56]
			FGF2	VEGFA, DKK1, SB431542		[57]
				Dorsomorphin		
				IWR1	Tri-iodothyrinine	[58]
Monolayer Differentiation	Monolayer on Matrigel with MEF	RPM1 plus B27	Activin A, BMP4	RPM1 plus B27	RPM1 plus B27	[59]
			Activin A, BMP4, FGF2	Noggin, RAi, DKK1	DKK1	[60]
		RPM1 plus B27(−insulin)	Activin A, BMP4, FGF2	VEGFA, DKK1	VEGFA, FGF2	[61]
	(KSR)/FGF2 on MEF	LI-APEL	Activin A, BMP4, FGF2, VEGFA, SCF	LI-BEL	LI-BEL	[62]
	mTeSR	RPM1 plus B27	Activin A, BMP4	IWR1 or IWP4	RPM1 plus B27	[63]
	mTeSR+ROCKi	RPM1 plus B27(−insulin)	CHIR	IWR1 or IWP4	RPM1 plus B27	
	Chemically defined E8	CDM3	CHIR/WNTC59	CDM3	CDM3	[65]

Table 2. Methods for hiPSC-CM differentiations (adapted from Smith et al. [54] and Burridge et al. [55]).

The first generation of CM differentiation was established using ESCs and successfully applied to hiPSCs. While the traditional embryoid body protocol engaged serum-derived spontaneous differentiation into CM, its efficiency was very low, (~1–5%). The second generation of CM differentiation aimed to recapitulate the embryonic developmental sequences *in vitro* by modulating stage-specific activation/inhibition of signaling pathways with recombinant protein, details described in **Table 2**. At the molecular level, each stage of iPSC-CM differentiation is characterized by sequential expression of specific sets of genes [53]. These protocols were much more efficient; however, they were expensive and exhibited high batch-to-batch variation.

The third-generation hiPSC-CM protocol is composed of sequential modulation of the Wnt signaling pathway: activation at an early stage with small molecules such as CHIR-99021 and then inhibition at a late stage with small molecules such as IWP2 [64–66]. These monolayer-based directed differentiation protocols generate CMs with high efficiency. On the other hand, maturation of these CMs became a major challenge for the use of *de novo* CMs in heart research, especially for disease modeling and drug testing [67, 68]. Thus, several studies used prolonged cell culture, electrical stimulation, mechanical stretch or hormonal stimulations to induce CM maturity [69–72].

hiPSC-CM technology has transformed the field of cardiovascular research, especially the study of inherited and acquired cardiovascular diseases. Several heart diseases including long QT syndromes, catecholaminergic polymorphic ventricular tachycardia, and familial hypertrophic cardiomyopathy have been modeled using hiPSC-CM [73–75]. Patient-specific CM regeneration may hold the promise for stem cell-based cardiac therapy.

3.2.3. Fallopian tube

Ovarian cancer is the leading cause of gynecologic cancer-related deaths in the United States. Fallopian tube epithelia (FTE) has been identified as the origin of ovarian cancer [76]. The discovery of serous tubal intraepithelial carcinoma (STIC) lesions, a preneoplastic finding in the fallopian tube fimbriae of patients with BRCA mutations, supports the model of FTE origin of serous "ovarian" carcinoma [77].

Yucer et al. [22] developed a hiPSC-derived 3D human FTE model, mimicking the FTE development process via various intermediate stages toward mature FTE in 3D organoid culture. Female reproductive tract structures including fallopian tube epithelium arise from the Müllerian duct in parallel to the urinary system from IM of the urogenital ridge in the posterior primitive streak. Therefore, Yucer et al. [22] recapitulated Müllerian development starting with IM generation and further developed into fallopian tube epithelial precursors using pro-Müllerian growth factors. Each step of this differentiation is monitored through the expression of established markers (**Figure 7**). Further differentiation of the fallopian tube epithelial lineage was attained on a 3D growth platform, which enables the FTE organoid to self-organize into a convoluted luminal structure with secretory and ciliated cellular components [22].

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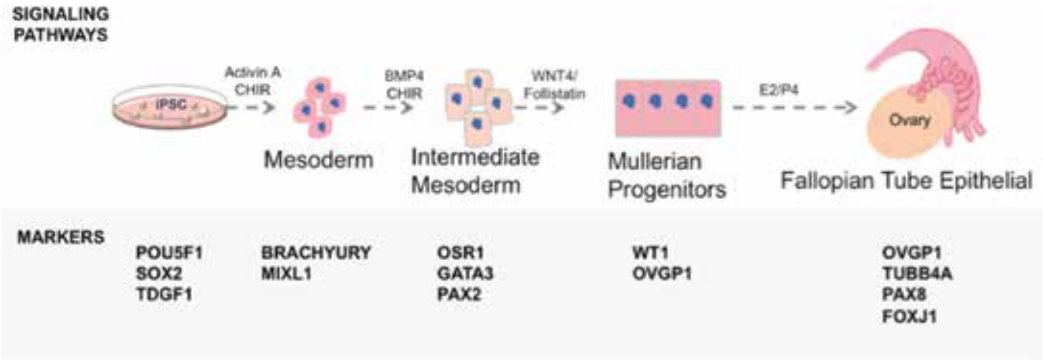

Figure 7. Schematic of iPSC derive FTE organoid model. The stepwise differentiation of FTE via various intermediate stages which are characterized by specific molecular signatures. BMP4, Bone Morphogenetic Protein bone 4; CHIR, CHIR99021; E2, Estrogen: P4, Progesterone; WNT, Wingless Type Family member.

hiPSC-derived 3D-FTE organoids model offers a faithful *in vitro* platform to investigate the fallopian tube origin of ovarian cancer and to explore early cancer pathogenesis and progression. This platform can also be used to study high-risk germline mutations including BRCA1/2, to identify the molecular signature and genetic alteration involved in carcinogenesis and ultimately uncover novel drug targets.

3.3. Endoderm-derived tissues

The endoderm gives rise to the epithelial lining of the gastrointestinal and respiratory tracts; the parenchyma of the tonsils, the liver, the thymus, the thyroid, the parathyroids, and the pancreas; the epithelial lining of the urinary bladder and urethra; and the epithelial lining of the tympanic cavity, tympanic antrum, and auditory tube (**Figure 8**).

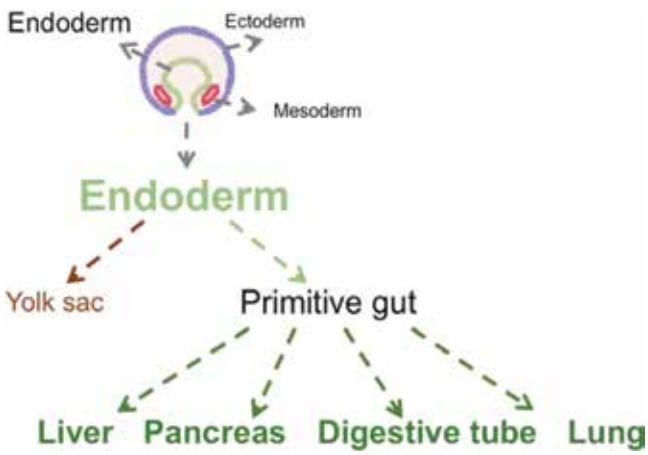

Figure 8. Summary of organs originated from endoderm.

3.3.1. Small intestine, stomach, and colon

The small intestinal tissue is composed of a single-layer of epithelial cells which form a lumen that is surounded surrounded by connective tissue. Functionally, the small intestine plays a central role in digestion and absorption of nutrients. There are different cell lineages (enterocytes, goblet cells, Paneth cells, tuft cells and enteroendocrine cells) in the small intestine with various functions such as exocrine, absorption, and protection. While diseases of the small intestine, such as tumor, inflammatory bowel disease (IBD), lactose intolerance are common, the study of these diseases have encountered difficulties due to the limitation of *in vitro* modeling systems.

The protocols used for intestinal organoid differentiation have been published by different groups [78, 79]. In general, hiPSCs are directed to differentiate into definitive endoderm followed by intestinal fate specification and development using Wnt3A and FGF4. Intestinal organoids, usually cultured in Matrigel, show a polarized, columnar epithelium that is patterned into villus-like structures and crypt-like proliferative zones that expresses intestinal stem cell markers. The epithelium contain functional enterocytes, as well as goblet, Paneth and enteroendocrine cells with a layer of mesenchymal cells. Yu et al. [80] reported a refined, non-Matrigel scaffold and 3D intestinal organoid culture protocol. The matrix-free system may improve the yield, decrease the time, and facilitate high-throughput approaches. The protocols used to generate intestines from hiPSCs are summarized in **Figure 9**.

Gastric ulcer and gastric cancer affect 10% of the world's population and there is no experimental model of the normal human gastric mucosa. The lack of proper models has hindered mechanistic studies, preventive approach testing, and disease modeling. Kyle et al. developed the first protocol directing hiPSCs to 3D gastric organoids by manipulating FGF, WNT, BMP, retinoic acid and EGF signaling pathways [81]. These organoids formed primitive gastric gland- and pit-like domains, proliferative zones containing LGR5-expressing cells, surface and antral mucous cells, and a diversity of gastric endocrine cells.

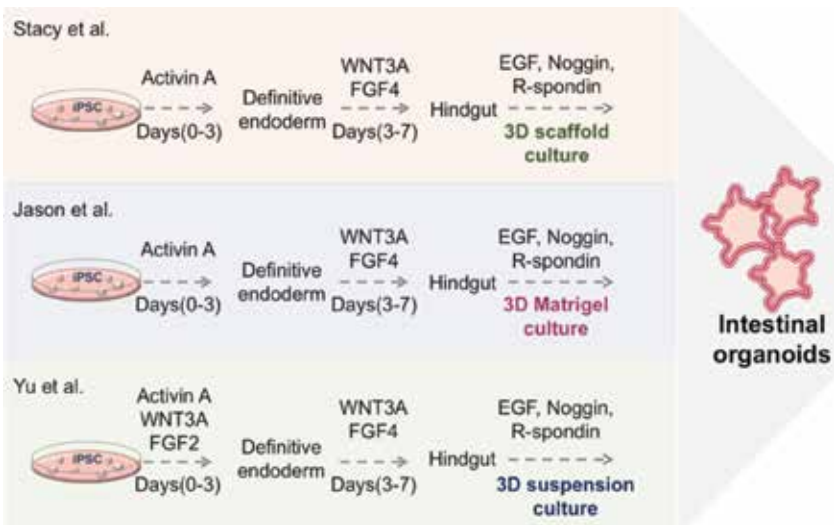

Figure 9. The protocols used to generate intestinal organoids using hiPSCs.

Recent studies have successfully generated colonic organoids from hiPSCs. Following the similar differentiation path to intestinal organoids, hiPSCs were sequentially differentiated into definitive endoderm, hindgut endoderm, and colonic organoids. The colonic organoid differentiation was conducted in a Matrigel 3D culture. Jorge et al. [82] modified the protocol used for small intestinal organoid differentiation by adding BMP2 in the Matrigel culture stage. BMP signaling can promote posterior fate in human gut tube cultures. Another group [83] reported a different approach by supplementing the inhibitors CHIR99021 and LDN193189, and EGF in the Matrigel culture stage. These hiPSCs-derived colonic organoids exhibit crypt-like structure formed by a polarized epithelium consisting of colon stem cells, goblet, and endocrine cells and a layer of supportive mesodermal tissue.

3.3.2. Lung

The regeneration of lung epithelial cells/organoids has applications in regenerative medicine, modeling of lung disease, drug screening and studies of human lung development. The lung is composed of endoderm-derived epithelial cells surrounded by mesenchymal-derived stromal cells. Lung epithelial cell differentiation follows the path of definitive endoderm to anterior foregut endoderm. Then the Nkx2.1+ endoderm will bud from the ventral side of the anterior foregut to form the primitive lung bud, which will form the respiratory tree. Signals from the mesenchyme to the epithelium are critical in cell specification, determination, and differentiation, and are essential for proper development and maturation of the lung [84].

A number of studies show the differentiation of hiPSCs into lung epithelial cells in 2D [84–88]. However, 3D lung organoid differentiation has become the trend. Briana et al. [24] reported a breakthrough in the stepwise differentiation of human lung organoids from hiPSCs that consist of both epithelial and mesenchymal components. These lung organoids possess upper airway-like epithelium with basal cells and immature ciliated cells surrounded by smooth muscle and myofibroblasts as well as an alveolar-like domain with appropriate cell types. Later, Chen et al. reported the generation of lung bud organoids (LBOs) that contain mesoderm and pulmonary endoderm and develop into branching airway and early alveolar structures after xenotransplantation and Matrigel 3D culture [89].

The application of acellular lung matrices has been reported in 3D lung tissue reconstruction. Decellularized lung matrix supports the culture and lineage commitment of hiPSC-derived lung progenitor cells [90]. The rotating bioreactor was also used to provide an air-liquid interface, which is a potent inducer of type I epithelial differentiation for both hiPSC-Alveolar epithelial type (AT) II and ATI cells [91]. The bioreactor system provides a method for large-scale production of alveolar epithelium for tissue engineering and drug discovery. Another improvement for lung regeneration from hiPSCs is the use of biomaterials [25].

4. Application of iPSC-based tissue regeneration

hiPSC-derived cells or organoids are becoming promising resources for disease modeling and therapeutical applications. In general, somatic cells from patients can be reprogrammed to hiPSCs. In turn, patient-specific hiPSCs can be converted into target organs using established

protocols. These *in vitro* derived organs can be used for multiple purposes, including patient-specific disease modeling, drug testing, therapy screening, and transplantation.

4.1. Personalized disease modeling

The biggest advantage of the hiPSC technology lies in its patient-specific feature. hiPSC-derived 3D organoid models have recently emerged as a powerful tool to recapitulate and investigate the physiologically-relevant process of disease onset and progression *in vitro*. This model system leverages the self-renewal and multi-lineage differentiation capability of multipotent stem cells and their intrinsic self-organization regenerative ability to form 3D tissue architecture. Importantly, hiPSCs can be derived from patients with known hereditary genetic mutations that are associated with a higher risk of a particular disease. This provides a valuable approach to determine whether additional genetic alterations are needed to interact with the known mutations, thereby contributing to disease susceptibility, initiation, and progression [92].

Several hiPSC-derived, inherited human disease models have been used to reproduce cancers associated with those high-risk patients [93, 94]. A hiPSC-derived osteosarcoma model for Li-Fraumeni syndrome has yielded promising results in displaying disease pathogenesis and carcinogenesis events commonly found in relevant human cells [95]. Cystic Fibrosis (CF) is an inherit disease of secretory glands. Among all the organs, pancreas is the earliest and most severely affected organs impacted by CF. hiPSC-derived pancreatic epithelial cells can be used to study personalized CF development [96]. Kyle et al. [81] used hiPSC-derived gastric organoids to model the pathophysiological response of the human stomach to *H. pylori* infection. In addition, Miguel et al. reported using hiPSC-derived colonic organoids to model family APC mutation-associated colon cancer initiation [83]. More and more hiPSCs-based disease models will be established.

4.2. Therapeutic applications

4.2.1. Drug screening

Organoids differentiated from patient-derived hiPSCs can be used to build a screening platform to develop and validate therapeutic approaches. hiPSC-derived organoids have a line of features that make them suitable models. Using a defined protocol, hiPSC-derived organoids become an unlimited resource for a specific patient. The *in vitro* direction of organ differentiation allows the rapid and robust generation of organoids with identical features. Most importantly, the organoids are 3D based mini-tissues that consist of multiple cell types, and that recapitulate the tissue structures *in vivo*. Thus, the drug screening results are more applicable *in vivo*. As an example, hiPSC-based drug screening for Huntington's disease has been established [97] developed. The applications of hiPSCs that have been reprogramed from patients of heritable, genetic diseases has been summarized by Wonhee Suh in a review paper [98].

Biomimetic tissues on a chip have been developed for drug discovery [99]. Organ-on-a-chip is based on microfluidic technology and has been proposed as a novel cell-based assay tool in pre-clinical studies. Furthermore, the concept of body-on-a-chip, which is stands for multiple organs connected through microfluid devices, can mimic multiple interactions between organs [100]. Applying hiPSC research to the concept of organ-on-a-chip has provided a promising future for the development of the patient-specific body-on-a-chip [101]. Drug

screening is no longer a process that is limited by the responses of targeted organ, it can also provide an evaluation of systemic responses.

4.2.2. Gene therapy

The nature of the disease and desired genetic modification, efficiency and accuracy of gene repair methodology, as well as cell state will determine the success of gene therapy [102]. In theory, monogenic diseases dictated by a dysfunctional copy of the causative gene would be reversed by introducing a wild-type copy of the gene into cells [103, 104]. Over 80% of rare diseases are considered to have a genetic origin [105], which means the precise gene editing technologies can be practically used to correct these genetic factors. The application of genome editing technologies in therapeutic trials have been reported in many diseases, such as retinal diseases [106], lysosomal storage diseases [107], arthritis [108], and neurological disorders [109, 110]. In contrast, polygenic diseases that require simultaneous multiple alterations of the genome are more challenging to treat with gene therapy [111].

Gang et al. presented a highly efficient and reproducible protocol to edit the genome of hiPSCs through the combined use of the CRISPR/Cas9 RNA-guided nuclease and piggyBac (bacterial artificial chromosome) transposase [112]. Their method can result in efficient, targeted genome editing and concurrent "scarless" transgene excision. Satoru et al. reported using gene editing with engineered site-specific endonuclease technology to treat dominant-negative disorders by targeting only the mutant allele while leaving the normal allele intact [113]. Using precise gene editing technology to correct gene mutations from hiPSCs generated from patients combined with hiPSC differentiation into target cells/organs for transplantation provides an immense promise for the future of gene therapy (**Figure 10**).

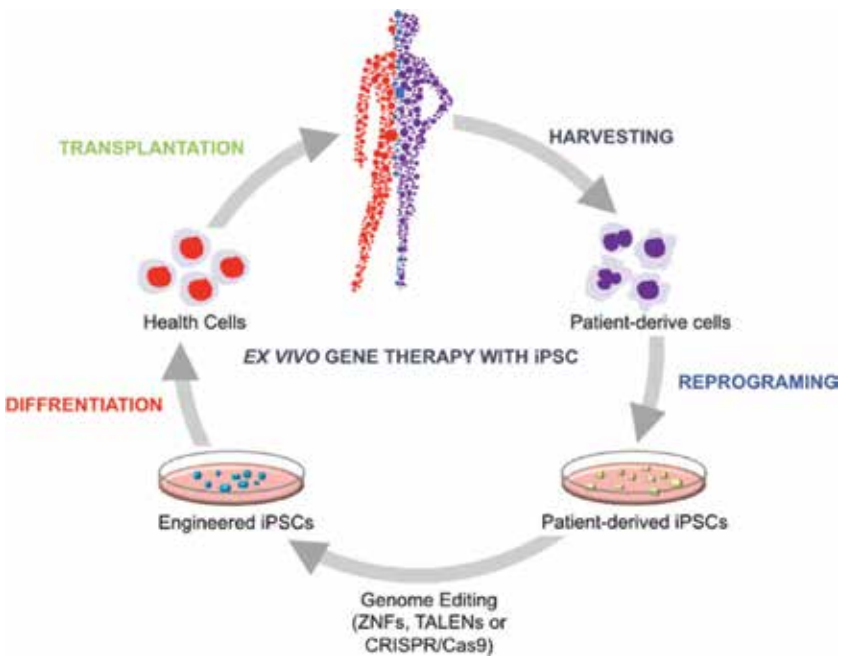

Figure 10. The summary of gene therapy applying precise genome editing technology in hiPSCs.

4.2.3. Transplantation

Given that hiPSCs are pluripotent stem cells which can be propagated unlimitedly and protocols for their differentiation into different cells/organoids have been established, hiPSC-derived micro-tissues are a potentially innovative material source for transplantation. In addition, immune rejection will be minimized when essentially returning the hiPSC-derived tissue to the original patient. For mature cells that have no or limited regenerative ability, such as cardiomyocytes, neurons, and pancreatic cells, hiPSC-derived cell/organoids are especially valuable for tissue repair. There are a series of clinical studies evaluating hiPSC-cells/organoids for treatment of neural degeneration, diabetes, heart failure, and retinal cells [114]. Although research on the application of hiPSCs in therapy have shown encouraging progress, there are some concerns involving the safety of hiPSC-based cell transplantation. Tumor risk and acquired gene mutations are major concerns.

5. Future and challenges

The original protocol to generate hiPSCs involves four transcriptional factors, but this method is not suitable because of its effect on genome integrity via the introduction of additional plasmids with exogenous genes. To make hiPSCs and their derivatives applicable for clinical uses, many improvements have been made to optimize the method for iPSC generation. The integration-free and chemical reprogramming protocols have been developed to minimize the risk of jeopardizing genome integrity [115, 116].

In general, the genetic nature of a disease, the molecular editing platform used, the delivery method, and the targeted cells and organs are all factors that influence the efficacy of treatment and determine the likelihood of clinical benefits [117]. The CRISPR/Cas9 molecular scissor system has been used to edit the genomes of a diverse array of mammalian cell types and organisms with high efficiency and precision. Determining and overcoming the actual frequency of off-target activities is challenging, yet critical to the application of the technology in gene therapy. CRISPR/Cas9 technology allows the study of complex genetic diseases, including human cancer, in which multiple mutations and chromosomal translocations are present in the genome [118, 119].

The potential application of hiPSC technology in cancer studies has been proposed, based on the idea of reprogramming cancer cells via hiPSC technology to cancer stem cell (CSC) state. CSCs are well-known as the origin of tumor development, the seeds for distant metastasis, and are critical in therapeutic resistance. Reprogramming the malignant cells back to their original state before the oncogenic transformation occurs [120], may provide tools for exploring the mechanisms of tumor initiation and progression *in vitro*, for studying the heterogeneity and origin of CSCs, and for producing cancer type-specific drug discovery. However, these reprogramming methods remain a challenge because of the cancer-specific epigenetic state and chromosomal aberrations of cancer cells.

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Conflict of interest

All the authors declare no conflict of interest.

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Chapter 8

Recent Advances in Stem Cell and Tissue Engineering

Farideh Mohammadian

Additional information is available at the end of the chapter

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Abstract

The clinical application of stem cells in tissue engineering and regeneration is becoming more significant. However, its application has been limited by issues like reproducibility of the stem cells, ethical concerns of harvesting some of these stem cells, and controlling the fate of stem cells in vitro and in vivo. The advent of tissue engineering and regeneration has led to the fabrication of advanced biomaterials and scaffolds with enhanced ability to mimic and control the cellular microenvironment similar to that of innate stem cell niche. Combining the use of stem cells with biomaterials and scaffolds especially synthetic hydrogels that have exhibited physicochemical abilities and properties similar to native niche can be the future of tissue engineering in terms of formation of new tissues like bones. Recently, there has an increase in the use of either endothelial progenitor cells (EPCs), induced pluripotent stem cells (iPSCs), or adult mesenchymal stem cells in preclinical studies: however this is yet to be transferred to clinical setups as there are limitations in terms of regulations and ethical considerations. The purpose of this review is to give comprehensive details about the application of stem cells in tissue engineering.

Keywords: endothelial progenitor cells, induced pluripotent stem cells, adult mesenchymal stem, tissue engineering, scaffolds

1. Introduction

Tissue engineering is a multidisciplinary science that applies the principles of bioengineering for the fabrication of new and improved biomaterials capable of maintaining and restoring the functionality of organs and tissues impaired by disease and trauma. This translational approach has been applied to develop and design patient-specific tissue grafts that mimic the functional properties of native tissues. Three important factors have been accredited to the success of tissue engineering: cocultured stem cells, signaling factor, and the bio-fabricated scaffold.

The stem cells are capable of differentiating into several types of tissues and organs, while the bio-fabricated scaffold provides structural support to the seeded stem cells. Signaling factors are responsible for influencing cell phenotype, metabolism, migration, and organization.

Stem cells are undifferentiated cells of embryonic, fetal origin, and they possess the ability to give rise to differentiated cells and then finally develop into organs. Stem cell characteristics include the ability to self-replicate and renew, clonage forming, and high potency ability [1]. In terms of the potency ability of stem cells, stem cells can be totipotent, could differentiate into any cell types (pluripotent) [2], and could differentiate into cells that arise from the three germ layers—ectoderm, endoderm, and mesoderm—from which organs develop [3].

Stem cells can be categorized broadly into embryonic and adult stem cells and are efficient cell sources for tissue regenerative applications. They have also been reported to have the abilities to promote tissue homeostasis, growth, and repair, thereby contributing importantly to tissue and organ regeneration [4]. Bio-fabricated scaffolds consist of decellularized biomaterials to provide structural and anatomical functions to the seeded stem cells, thereby resulting into successful formation of specific tissue. In support of the above report, Kang and colleagues demonstrated that decellularized scaffolds loaded with autologous adipose-derived stem cells (ADSCs) were efficient to repair cartilage defect in an animal model [5]. They concluded that decellularized scaffolds loaded with ADSC induced significant and improved cartilage tissue repair compared to native cartilage.

2. Mesenchymal stem cells seeded for bone tissue engineering

MSCs are stromal stem cells that are heterogeneous and are derived from several tissue sources that include adipose tissue [6], periodontal ligaments [7], bone marrow (**Figure 1**) [8], umbilical cord (UC) [9], placenta [10], and lungs [11]. MSCs express surface markers like CD73, CD44, CD90, and CD105. The most widely known and used MSCs are bone marrow MSCs and adipose tissue-derived MSCs isolated and purified from the bone marrow and adipose tissue, respectively. Briefly, the anatomy of the bone marrow is made up of the parenchyma and the stroma part. The parenchyma houses the hematopoietic stem cells, and the stoma part consists of the bone marrow stromal cells (MSCs) that have the capability to differentiate into several cell lines like osteoblasts, chondrocytes, adipocytes, etc. The clinical use of both bone-derived mesenchymal stem cells and adipose stem cells in bone tissue engineering has been reported using various models of bone regeneration such as osteogenesis [12, 13], long bone defects [14, 15], and calvarial defects [16, 17]. Furthermore, co-administration of stem cells with cytokines has been reported to be efficient in bone repair as cytokines and growth factors like stromal derived factor-1 (SDF-1) can lead to increased migration and homing of stem cells to the defected site [18]. In a similar report by Ho et al., they demonstrated that co-administration of stromal-derived factor-1 with BM-MSCs would indirectly enhance bone repair by improving migration of innate cells to the site of bone fracture. They concluded that BM-MSCs overexpressing SDF-1 were efficient in improving the new bone formation during the early stage of fracture healing compared to BM-MSC treatment alone [19]. Genes implicated in fracture healing such as osterix [20], hypoxia-inducible factor-1 [21], and BMP-7 [22] have all been reported to be efficient in bone formation when transfected with MSCs.

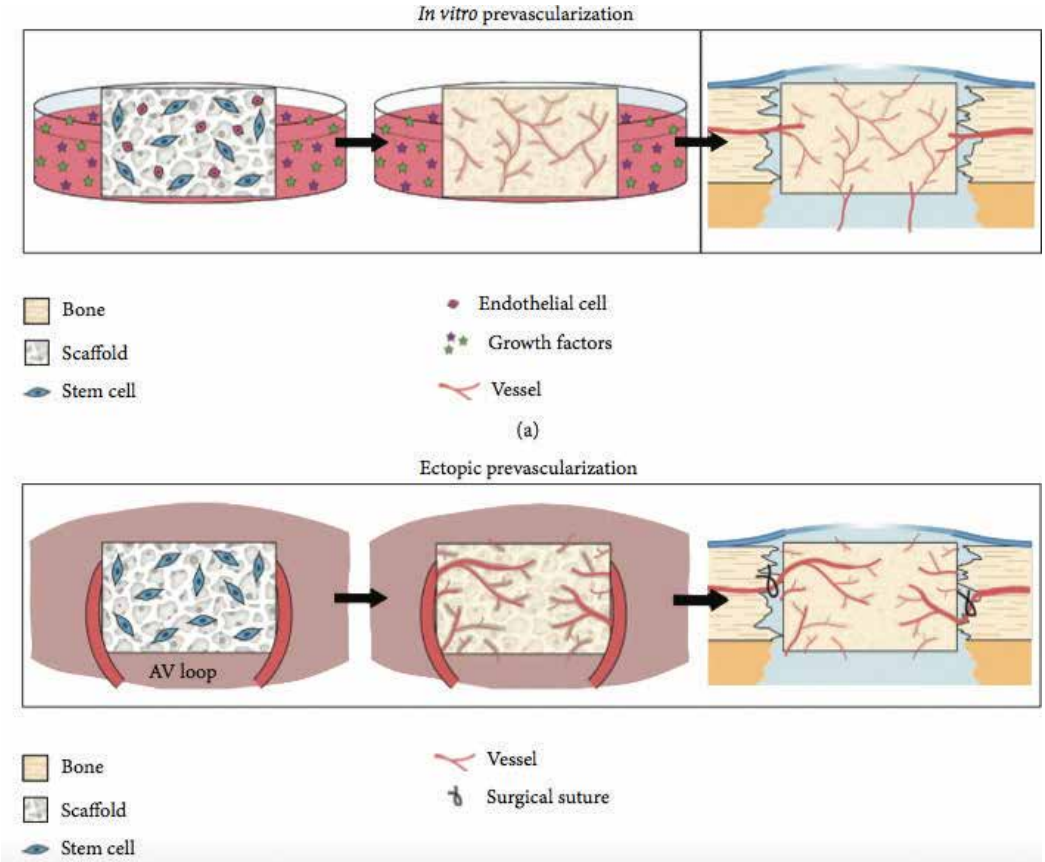

Figure 1. Showing in vivo and in vitro stem cell application in engineered tissue (a) In vitro prevascularization methods induce cell-seeded scaffolds to form vasculature (b) In vivo ectopic prevascularization involves implantation of a cell-seeded scaffold into a highly vascularized bed. Adapted from [26] with copyright permission.

3. Advances in MSCs and tissue engineering technology

Recently, bone tissue engineering in combination with novel stem cell-based technologies is yielding promising results as reported by Syed-Picard and colleagues in their experimental study that BM-MSC-derived cell sheets could be used to fabricate functional periosteal tissue [23]. Briefly, culturing BM-MSCs to hyperconfluence to produce abundant extracellular matrix to form robust cell sheets generated the BM-MSC-derived cell sheets. The authors reported that the generated cell sheets supported with calcium phosphate pellets were transplanted subcutaneously into mice for 8 weeks. They concluded that there was significant bone-like tissue formation by the BM-MSC-calcium phosphate pellet structure compared to the non-seeded calcium phosphate scaffold.

In another similar study by [24], BM-MSC cell sheet technology was compared to control cell complex. The authors reported that BM-MSC cell sheet resulted into significant expressed levels of growth factors crucial to bone development like vascular endothelial growth factor and PDGF. In another innovative study of stem cell application in tissue engineering, Ren et al. fabricated

and demonstrated a three-dimensional vascularized stem cell sheet construct, composed of both BM-MSCs and human umbilical vein endothelial cells. The authors concluded that there was significant formation of blood vessel formation compared to the control [25].

4. Clinical reports on stem application of bone tissue engineering

A clinical study by Centeno and colleagues reported the application of culture-expanded, autologous BM-MSC for osteoarthritis in more than 300 patients. The authors reported the safety and efficacy of its use. Briefly, the autologous BM-MSCs were cultured in monolayer culture flasks and transplanted into the affected peripheral joints. They concluded that a 50% improvement in clinical symptoms was recorded among the osteoarthritis patients [27]. The application of infrapatellar fat pad-derived ASCs was demonstrated by Koh and colleagues when they reported its efficiency in improving and managing knee osteoarthritis through clinical and radiological results [28].

A mixture of stem cells and progenitor cells with CD90- and CD14-expressing cells resident in the bone marrow called tissue repair cells (TRC) has been demonstrated to be efficient for reconstructing craniofacial bone defects in a controlled feasibility trial [29]. Briefly, the clinical trial was carried out using 24 patients in need of localized osseous reconstruction. The patients were randomized to either guided bone regeneration (GBR) or TRC transplantation and were subsequently assessed. They concluded that TRC therapy resulted in an accelerated and improved alveolar bone regeneration compared to GBR therapy.

5. Induced pluripotent stem cells seeded for bone tissue engineering

Yamanaka's group is one of the pioneers of studies related to induced pluripotent stem cells. Yamanaka et al. studies like [30] where they reported the possibility of reprogramming of somatic cells into a primordial embryonic stem cell-like state, capable of differentiating into all three germ layers. There are several studies demonstrating the application of iPSCs in tissue engineering like [31], where they reported the ability of polyethersulfone scaffolds seeded with iPSCs to regenerate cranial bone. The authors concluded that iPSCs seeded with polyethersulfone scaffolds promoted and stimulated cranial bone formation compared to scaffold alone. In similar scaffold study design, Liu used an Arg-Gly-Asp-grafted calcium phosphate cement scaffold seeded with iPSC-MSCs overexpressing NELL1 that were efficient to improve osteogenic differentiation process [32]. However, this report was challenged by [33] reporting that osteogenic abilities of iPSCs can only be realized by scaffolds fabricated with calcium phosphate alone in an ex vivo model. The use of iPSCs in tissue engineering has been reported using animal model by Lian and colleagues, in their mouse model of limb ischemia study. They reported that iPSC-MSCs were more efficient compared to adult BM-MSCs [34] based on their more efficient survival and engraftment abilities after transplantation to induce tissue regeneration.

6. Endothelial progenitor cells (EPCs) seeded for bone tissue engineering

EPCs are bone marrow-derived precursor cells and express CD34 molecules. They have the ability to differentiate into endothelial cells and ultimately contribute to the process of angiogenesis [35]. They have been reported to be resident cells in the peripheral blood and potentially contribute to the initiation of neovascularization [36]. There have been several studies demonstrating the use of EPCs in tissue engineering. Zigdon-Giladi and co-workers in their nude mouse model study with calvarial defect demonstrated that human EPCs could enhance the processes of vasculogenesis and osteogenesis [37]. They concluded that there was a significant increase in blood vessel density as well as increased extra-cortical bone height and length in the human EPC-transplanted group compared to the control. Furthermore, EPCs seeded on Gelfoam scaffold were reported to be efficient in stimulating cranial bone formation at the site of injury compared to the unseeded scaffold [38].

In a clinical case carried out by Kuroda and colleagues of tibial surgery, the efficacy of EPCs was demonstrated when autologous, granulocyte colony-stimulating factor (GCSF)-mobilized CD34(+) cells were used in successful tibial autologous bone grafting [39].

7. Stem cells and decellularized scaffolds

Recently, scaffolds have been designed in the form of decellularized tissues and organs and are commonly used in tissue engineering and regenerative medicine (**Table 1**). Recent and novel advancement in tissue engineering has been the bedrock for the functional replacement of whole organs. Several organs have been bioengineered and implanted into laboratory animal recipients and potentially showing regenerative abilities and functions. Both acellular and decellularized scaffolds have been seeded with stem cells and potentially have exhibited promising clinical results.

Organ/tissue engineered	Decellularized scaffolds	Cells	Type of experiment	References
Skin tissue engineering	MatrACELL-processed human acellular dermal matrix	NA	In vivo and clinical	[40]
Urethral tissue engineering	3D porous urinary bladder acellular matrix	NA	Clinical	[41]
Bone tissue engineering	Decellularized bone cylinders	Human-induced pluripotent stem cells	In vitro and in vivo	[42]
	Decellularized bone scaffolds	Human adipose-derived stem cells	In vitro	[43]
Cardiac tissue engineering	Decellularized porcine pulmonary valves	Autologous bone marrow mesenchymal stem cells	In vivo	[44]

Organ/tissue engineered	Decellularized scaffolds	Cells	Type of experiment	References
Pulmonary tissue engineering	Acellular porcine and human trachea-lung scaffolds	Murine embryonic stem cells; bone marrow-derived mesenchymal stem cells	In vitro	[45]
Renal tissue engineering	Decellularized rat kidneys	Mouse embryonic stem cells	In vitro	[46]
Tracheal tissue engineering	Genipin cross-linked decellularized rat tracheal scaffold	Pluripotent murine embryonic stem cells	In vitro	[47, 48]
	Decellularized leporine tracheal scaffold	Bone marrow-derived mesenchymal stromal cells	Clinical	[49]
Vascular tissue engineering	Decellularized vascular scaffold from rat abdominal arteries	NA	In vivo	[50]
	Decellularized inferior vena cava of rabbits	Adipose-derived mesenchymal stem cells	In vitro	[51]
Neural tissue engineering	Genipin cross-linked gelatin electrospun scaffolds	Rat allogeneic mesenchymal stromal cells	In vitro	[52]
Cartilage tissue engineering	Decellularized stem cell matrix	Human adult synovium-derived stem cells	In vitro	[53]
Hepatic tissue engineering	Acellular whole liver scaffol	Mesenchymal stem cells	In vitro and in vivo	[54]

Adapted from Rana et al. [55] with copyright permission.

Table 1. Applications of tissue-engineered scaffolds recellularized with stem cells.

8. Decellularized cardiac tissues and stem cells

There are reports of fabricated decellularized cardiac tissue used as potential scaffolds. For example, human cardiac extracellular cell matrix sheets have been reported to be seeded with mesenchymal stem cells and cardiomyocytes by [56]. The authors concluded that the MSC-cardiomyocyte-derived scaffold efficiently improved and stimulates cardiac tissue regeneration. In another report by [57], decellularized engineered heart valve was successfully implanted for reconstruction of the right ventricular outflow tract. The authors concluded that via echocardiography, the implanted heart valve demonstrated normal physiological pressure gradient after 10 years, with no record of calcification and, in addition, it exhibited an excellent hemodynamic performance.

9. Decellularized respiratory tissues and stem cells

There are reports of fabricated decellularized tissue used as potential scaffolds for respiratory tissue because of their simplified anatomical structure. One of the earliest reports on the fabrication of scaffold for tracheal tissue regeneration is [49] where the authors compared the efficiency of decellularized leporine tracheal scaffold seeded with amniotic-derived mesenchymal stem

cells and non-seeded decellularized scaffolds. The authors concluded that MSC-seeded scaffold exhibited a high level of survival of the cells and epithelialization as well as a high level of elastin.

In another study on decellularized tissue, Nichols and colleagues fabricated acellular pig scaffolds using decellularized scaffold seeded with murine embryonic stem cells, pig bone marrow-derived mesenchymal stem cells, and primary human alveolar epithelial type II cells [45]. They concluded that there were recorded changes in type I collagen levels and evidences of cell attachment and viability.

10. Clinical application of tissue-engineered trachea and stem cells

There have been some reports on the successful implantation of bioengineered tissues like tracheal seeded with stem cells clinically. Macchiarini and colleagues first reported the fabrication of human tissue-engineered trachea seeded with autologous epithelial cells and mesenchymal stem cell-derived chondrocytes. They reported that the engineered scaffold was later transplanted into a bronchomalacia patient to replace her left main bronchus. They concluded that there were evidences of functional airway activities and improved mechanical properties of the scaffold within 4 months [58].

In another clinical report by Otti and co-workers following a 5-year study, transplanted tracheal graft exhibited excellent vascularization and recellularization with respiratory epithelium and normal ciliary functions [59]. However, the authors also reported that because of longer production period of the tracheal graft, it might not be suitable for patients in need of urgent transplantation. In a quest to produce a tracheal graft with reduced production time, Baiguera and colleagues designed a human tracheal graft with production period of 3 weeks. The authors reported that the fabricated graft still possess structural and mechanical properties similar to native trachea [47].

In another innovative clinical study carried out by [60], the authors replaced an adult airway with a stem cell-seeded decellularized tracheal scaffold in a patient suffering from congenital tracheal stenosis. They concluded that the graft scaffold showed accelerated revascularization followed by epithelialization after 12 months. Recently, human-derived decellularized trachea seeded with stem cells was demonstrated to be efficient in terms of stability, epithelialization, neovascularization, and chondrocytes formation in a patient suffering from tracheal stenosis [61].

11. ASCs and breast tissue regeneration

Adipose tissue is an important constituent of soft tissues in the body that offers protection to underlying structures. Tissue flap procedures are said to be more efficient in producing a more natural reconstruction; however it is very invasive, while breast implants have been associated with complications like extrusion and lack of contraction of the breast capsule. Adipose-derived stem cells (ASCs) have been identified to be the leading candidate for breast reconstruction, although ASC supplementation has been studied in clinical trials for wound healing therapies [62].

12. Fat graft and ADSC application for breast tissue regeneration

Masuda and colleagues [63] in their report demonstrated that transplanted omental tissue both in the presence and absence of pre-adipocytes isolated from epididymal adipose tissues under the dorsal skin of Wistar rats after 12 weeks efficiently produced high levels of triacylglycerol content, capillary density, and VEGF. They concluded that co-transplantation with pre-adipocytes significantly accelerated adipose tissue formation. In another study by Matsumoto and co-workers [64], they reported that cell-assisted lipotransfer (CAL) fat had an increased survival rate than non-CAL fat, and there were early signs of microvasculature in CAL fat. Moseley and colleagues [65] using a nude mice showed that fat supplemented with ASCs sustained its adipocyte-rich appearance and weighed 2.5× greater compared to non-ASC supplemented grafted fat. Furthermore, Zhu and co-workers reported comparable findings that fat grafts treated with ASCs increase capillary density and neovascularization [66]. Several studies have also reported that cultured human ASCs produce and release several angiogenic growth factors under hypoxia condition [67, 68] and have been associated with increased fat graft microvasculature.

Coleman and colleagues in a retrospective study of 17 breast procedures done from 1995 to 2000 reported that all patients had a significant enhancement in their breast size and shape postoperatively [69]. Coleman and colleagues stated that most patients in their study underwent mammography a year after breast surgery without any known screening complications. Yoshimura and colleagues [70] in their clinical study did CAL on six patients with facial lipoatrophy. The authors concluded that the CAL group had a better clinical improvement score compared to the non-CAL patients. In another related study by Yoshimura and colleagues [71], they conducted two clinical trials, using CAL for breast reconstruction. Yoshimura et al. concluded that after treatment of 55 patients, there was advancement in the clinical results with evidences of graft retention. In addition, reconstruction and retention outcomes were demonstrated by Kitamura and co-workers [72] after CAL treatment in five patients.

In a more recent clinical study by Tissiani and Alonso [73], they investigated the effectiveness of autologous fat grafts supplemented with stromal vascular fraction (SVF) in secondary breast reconstruction surgery. The authors concluded that after 3 years of follow-up of the patients they proved volumetric persistence of this type of fat tissue grafts without any significant clinical complications recorded. In another clinical study by Claro and colleagues, it was reported that the complication rate after autologous fat tissue grafting was low compared to the complication rate after breast reconstruction surgery procedures done with breast implants and/or myocutaneous flaps [74].

13. ASCs and skin tissue engineering

Böttcher-Haberzeth and colleagues have extensively reported the development of a skin substitute known as tissue-engineered dermo-epidermal skin substitutes (DESS). It is made of the two basic native skin layers, epidermal and dermal layers, and it can potentially serve as a

near replacement for the natural skin for clinical application [75]. Adipose-derived stem cells are attractive and valuable tools for regenerative skin engineering as they can differentiate into different skin cell lineages as well as secrete paracrine factors responsible for initiating skin tissue repair and regeneration.

Trottier and colleagues demonstrated the endogenous production of the extracellular cell matrix components by various skin cells known as IFATS collection. The authors reported that through this method there was formation of strong multiple layers of cell sheet that lead to increase in the skin graft thickness. The authors recorded satisfactory epidermal thickness and stratification [76]. In another study by [77], the authors seeded ASCs onto different scaffolds to determine the differentiation fate of the respective cells. The ASCs seeded on collagen type 1-based matrix and PEGylated fibrin-based scaffold differentiated into fibroblast-like dermal cells and blood capillary network, respectively. Recently, tropoelastin-based scaffold for skin substitutes was developed by [78]. Briefly, biomimetic scaffold was seeded in vitro with ASCs and transplanted onto the SCID mice. The authors concluded that ASCs grew rapidly and colonized the scaffold that resulted in increased epidermal thickness in vivo.

14. Conclusion

Scaffold-based tissue engineering using stem cells has improved the field of tissue regeneration in medicine; however, it is still at the infancy level. An extensive in-depth scientific knowledge and study of different stem cells will go a long way to translate them to clinical application. In addition, more extensive studies are needed to be done on different scaffold designs because the success of tissue engineering depends on these scaffolds and provides a niche to transplanted cells. Furthermore, most of the use of stem cells in tissue regeneration has been directed toward small tissue defect as such efforts to develop bioengineered grafts to repair larger tissue defects (bone defects) should be made. Several stem cells like induced pluripotent stem cell, mesenchymal stem cells, and ASCs are promising source of patient-specific stem cells with great regenerative potential. However, few or no clinical translation is available as they are potential teratoma and carcinogenic causative agents, and isolation of some of these cells is deemed unethical. Stem cells seeded on decellularized scaffolds have been reported to demonstrate promising and excellent results over the years. However, more clinical evaluations are needed to be properly sure they are safe clinically.

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Section 5

Hard Tissue Regeneration

Chapter 9

Hard Tissue Regeneration Treatment Protocols in Contemporary Oral Surgery

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Additional information is available at the end of the chapter

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Abstract

Dental implant placement is one of the most reliable and predictable treatment choices in modern oral surgery. It requires available bone volume to resist the force during loading. There are many ways to regenerate the bone to place the implants with the desired dimensions. Guided bone regeneration, socket grafting, allograft bone block grafting, and intra- and extraoral autogenous bone block grafting are the most popular treatment approaches to reconstruct hard tissues. Autogenous bone graft is still considered the gold standard for the reconstruction of hard tissues. In addition, there are many scaffold biomaterials available that are used as templates for new bone formation. These biomaterials are helpful to not only eliminate the usage of autogenous bone grafts but also decrease patient morbidity. Another advantage of biomaterial usage in tissue regeneration is to reduce the learning curve of treatments by facilitating operative approaches. The aim of this chapter is to evaluate contemporary biomaterials that are used to reconstruct hard tissue defects in oral surgery.

Keywords: hard tissue engineering, bone defect, biomaterials, scaffold, dental implant

1. Introduction

The main reasons for tooth defects include periodontal diseases, decay, trauma, failed endodontic treatments, congenital anomalies, oncologic diseases, oral infections, and orthodontic treatment [1]. Functional and esthetic treatment for tooth loss is important but time-consuming. Dental implant applications are among the many methods developed to treat this problem [2]. Since the definition of osseointegration, dental implants have been a proven and frequently used method in the treatment of total and partial tooth loss [3, 4].

The success of dental implants is assessed by criteria such as implant survival, stability of prosthetic treatment, radiological bone loss, and presence of peri-implant infection [5]. The accepted general consensus for the success of dental implants in recent years is that both functional and esthetic results are satisfactory [6]. There are risk factors that should be considered for the success of an accomplished outcome. Some of these factors include age, sex, general health status, habits, the region where the implant is placed, the number of implants, and the condition of the bone [7].

For dental implant indications, the presence of adequate bone and the relationship between both jaws are important. Studies have reported that a non-ideal three-dimensional implant placement may cause peri-implantitis, esthetic and functional failure, and may even result in removal of the implant [8]. To achieve optimal esthetics and function, the position of implant in the alveolar crest has to be in a biologically correct and prosthetically driven location [9]. When the implant is placed in an inappropriate position, for example, a bone-directed position, the use of pink porcelain and/or angulated abutments would be inevitable. Besides, non-axial masticatory forces will increase the risk of complications, such as screw loosening or fracture and chipping on implant-supported restoration [10]. Insufficient alveolar ridges may require bone augmentation procedures to achieve optimal bone volume before implant placement. These applications ensure that the implant is placed in the correct position and that an appropriate restoration can be performed [11].

The amount and location of bone resorption are important factors in the selection of the augmentation technique. In addition, the relationship between the jaws in radiological and clinical evaluations should be considered in the sagittal, frontal, and transverse planes [12]. Alveolar bone augmentation procedures include applications for increasing residual crest width and/or height using grafts and/or biomaterials or for optimizing bone contours with repair of bone defects [13]. In an attempt to correct bone defects, many techniques have been extensively described for bone augmentation and grafting materials. Although autografts remain the "gold standard," the use of biomaterials in orthopedics and dentistry is increasing [14].

2. Bone augmentation

Bone augmentation procedures usually involve bone block grafts, guided bone regeneration, ridge expansion/splitting, sinus floor elevation, and distraction osteogenesis. In addition, socket preservation is often used for the protection of the existing bone. Despite the availability of these techniques, guided bone regeneration has been widely used for implant site development [15]. This is attributed to its predictability, easiness while handling, and less-invasive nature than other advanced bone augmentation techniques [16]. Another advantage of this procedure is that it can be performed prior to or simultaneously with implant placement [17]. The results of horizontal bone augmentation are more reliable than those of vertical bone augmentation. Achieving bone gain in the vertical dimension is more difficult than that in the horizontal dimension [18].

Using a bone graft does not always guarantee clinical success. There are many major and minor factors that affect clinical success [19].

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Major factors:

- Patient selection, patients without medical problems
- Defect morphology: multiwalled bone defects
- Graft types: autografts are preferred for allografts and allografts are preferred for alloplasts
- Healing capacity of the patient

Minor factors:

- Flap design
- Graft placing method
- Epithelial retardation

3. Bone graft healing mechanism

The main component of bone healing is the selection of the materials for the bone graft. Bone grafts have different bone-forming capacities; therefore, we need to understand the mechanisms of bone regeneration for the grafts used at the recipient regions. The requirement of the region can be determined in advance and the graft is chosen accordingly. Bone healing in the region where the graft is placed is supported through osteogenic, osteoconductive, and/or osteoinductive mechanisms.

3.1. Osteogenesis

Osteogenesis is defined as the formation of bone in the region where osteoblasts and osteoblast precursors do not have bone tissue. New bone formation occurs when osteoblasts and osteoblast precursors are produced by cancellous bone and bone marrow. Osteogenesis (bone formation) is characterized by the presence of living osteoblast cells in the graft material. The only bone graft with osteogenesis is the autogenous bone [20]. Autogenous bone grafts, also called autografts, are grafts transplanted from one site to another. The most effective type in terms of osteogenesis is cancellous bones, due to the migration of bone cells at high concentrations. Autografts have been observed to have bone formation capacity even when bone tissue is placed underneath the skin [21]. Vascularization of the graft site is necessary for continued osteogenesis. Some studies have reported loss of osteogenic properties of free autogenous grafts without vascular support within 5 days and that they continued osteoinductive and osteoconductive effects at the end of the study [20, 21]. Therefore, free autogenous bone grafts show osteogenic characteristics only for a few days. We should pay attention to the viability of the cells when placing the autogenous graft in the recipient region. Once the autogenous bone has been obtained, it should not be left in the dry area, and if possible, it should be used as soon as possible with saline in a sterile environment [22].

3.2. Osteoinduction

Osteoinduction is an active process in which the bone graft causes the bone-forming cells to penetrate the recipient region and stimulates them to form new bones. Osteoinduction refers to the ability of the graft to send a signal to attract, proliferate, and differentiate early-lineage cells (e.g., mesenchymal stem cells or osteoprogenitor cells) into bone-forming cells, resulting in the formation of a mineralized bone. Bone morphogenetic proteins (BMPs) support these signals. BMP is measured as the amount of picograms in the normal bone. In recent studies on osteoinduction, Urist et al. isolated BMP, a soluble glycoprotein. They described BMP as a growth factor of the transforming growth factor (TGF)-β family and as an inductive agent. They also reported that at least 15 different types of BMPs were found, and the most important were BMP-2 and BMP-7 [23]. BMP is naturally released during trauma or the regeneration process and acts as an osteoinductive agent.

Demineralized bone matrix (DBM) allograft materials have osteoinductive healing mechanisms. DBM allografts can provide a matrix for bone cells to infiltrate and produce bone. Its healing mechanism manifests through osteoinductive pathways, and bioactive molecules stimulate mesenchymal cells to differentiate into bone-forming cells [24].

3.3. Osteoconduction

Osteoconduction is described as the growth of a superficial bone on a surface. Osteoconductive materials are biocompatible and have an osteoconductive surface: on its pores, in its ducts, or in its tubes. Materials with osteoconductive properties form a matrix and guide osteogenesis. Grafts with osteoconductivity have no bone formation capacity and can only function as a roof for bone formation. If osteoconductive materials are placed in ectopic areas such as subcutaneous bones, bone formation does not occur and the material remains unchanged or resurfaced [22]. Examples of osteoconductive properties are autografts, allografts, xenografts, calcium sulfates, calcium phosphate cements, ceramics, collagen, and synthetic polymers. It is also known that bone graft materials may be supplemented with materials such as exogenous growth factors, to create inductive effects [22].

3.4. Creeping substitution

Creeping substitution indicates the movement of new tissues through channels made by blood vessels invading a transplanted bone. The dynamic healing and reconstructive process of bone transplantation was described by Axhausen in 1907; he reported that bone transplants undergo necrosis. The necrotic bone is then replaced by the new bone via creeping substitution [25].

Improvement of the graft material differs according to graft type in terms of duration and content. Vascular support in the recipient region and the survival rate of cells in the graft have a direct impact on graft recovery. Morphologically, the cortical bone, which is the tight structure around the haversian and Volkmann channels, consists of circular, parallel, and interstitial bone lamellar. The cancellous bone is porous and trabecular in shape and contains the bone marrow. There is a less surface area in the cortical bone than in the cancellous bone; therefore,

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the cells and blood vessels can reach the receiving region. The vascular support in the organization of the cancellous bone in the graft is 30% better than that in the cortical bone [26].

4. Bone augmentation techniques

4.1. Sinus lifting

Prostheses that are supported on maxillary dental implants are now the optimum way to give patients an admissible quality of life. In cases with a vertical insufficient alveolar bone, a maxillary sinus lift with a bone graft using a crestal or lateral approach is needed. Elevation of the sinus floor permits the correct number and length of endosseous implants to be applied for adequate mechanical support of the atrophic posterior maxilla [27].

Previous studies proved that dental implants related to maxillary sinus augmentation have a satisfactory long-term success and survival rate [28]. Implant application may be simultaneously combined with maxillary sinus lifting procedure as a" one-stage" surgery, or sinus lifting may be conducted at first, and implants are then applied as a" two-stage" operation. There are many options for graft material to augment the maxillary sinus. Autogenous grafts can be harvested from the chin and ramus intraorally or iliac crest, calvarium, and tibia extraorally. The disadvantages of autogenous grafts are resorption rate and morbidity. Allografts (cadaveric bone) are harvested and different techniques such as irradiation and freeze-drying are used to reduce antigenicity. Allografts are found in tissue banks. Xenografts consist of anorganic bovine or equine bone. The organic components of these types of grafts are chemically removed and a mineral scaffold is obtained. Alloplasts are synthetic materials; there are many types of structures of alloplastic grafts such as micro- or macroporous, dense, amorphous, or crystalline grafts. Structure and porosity directly influence the performance of the material [29].

4.2. Socket preservation

Following tooth extraction, alveolar bone remodeling begins by means of vertical and/or horizontal bone resorption [30] so that a proper prosthetic and esthetic position of dental implants can be influenced. Alveolar socket preservation techniques have been introduced to conserve the alveolar bone vertically and horizontally [31].

Socket preservation could be considered when:

- Implant placement needs to be delayed for patient- or site-related reasons;
- In cases where implant placement needs to be postponed for >6 months for some reason; and
- If partially fixed pontic site is planned [32].

There are various graft materials used in socket preservation surgery such as autografts, allografts, xenografts, alloplasts, or platelet concentrates. Allogenic bone is described as the

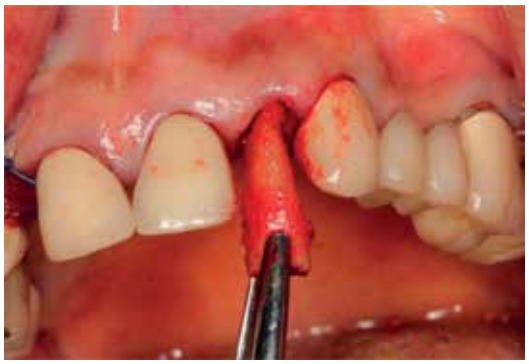

Figure 1. Extraction of lateral incisor.

most suitable material to obtain optimum results for socket preservation techniques. Freeze-dried bone allograft (FDBA) and demineralized freeze-dried bone allograft (DFDBA) are used in socket preservation techniques. Recently, platelet concentrates have been widely used for socket preservation. The platelet concentrates contain a high concentration of growth factors, such as PDGF, TGF-β, IGF, and VEGF, as well as anti-inflammatory molecules, such as IL-1β, IL-4, IL-6, and TNF-α, which accelerate the healing process. This results in better bone repair and regeneration [33].

Primary closure of the flap is important and should be performed if possible. The other methods to seal the surgery site are free gingival grafts, collagen membranes, or nonresorbable membranes [34]. The socket-shield technique is currently performed. Applying this technique, a buccal part of the tooth root is retained in the alveolar socket during tooth extraction. This is done to prevent the resorption of the vestibular bony lamella [35].

Several studies have reported that the socket preservation technique is very successful and useful compared to nongrafted sockets [31]. If immediate implantation is not possible, the socket preservation technique should be used to increase esthetic outcome as well as alveolar bone quality (**Figures 1–4**) [35].

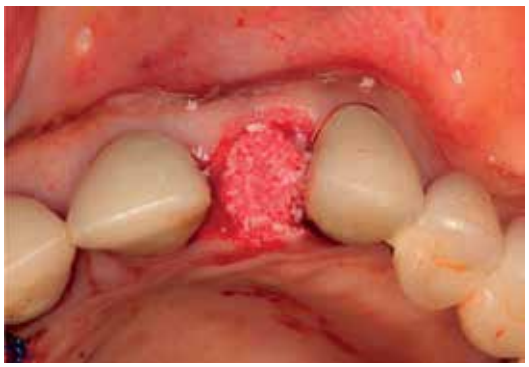

Figure 2. Applying of bone graft material.

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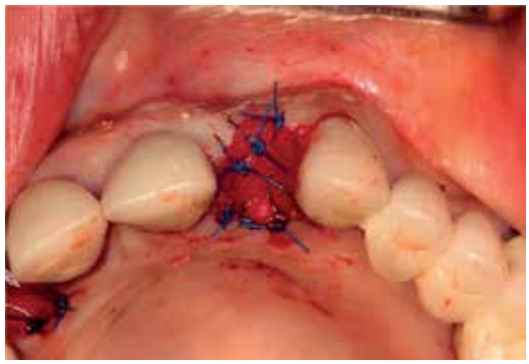

Figure 3. Sutures and closure.

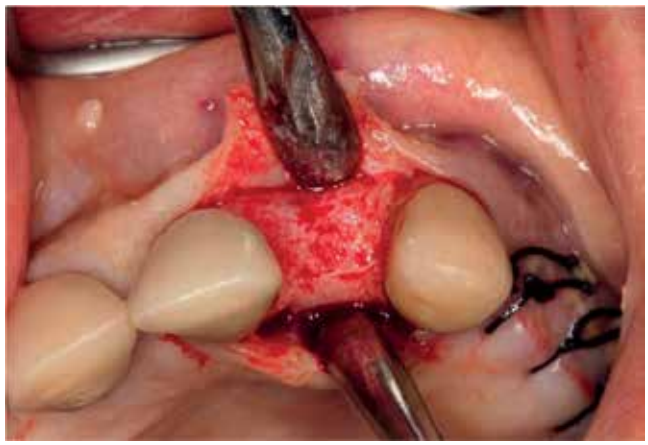

Figure 4. Post-op 6 months.

5. Types of bone grafting materials

Graft materials may be synthetic or natural materials that are placed in a biological environment for reconstructive purposes, and are prepared to be accepted by the surrounding tissues. The most commonly used biomaterials include autografts, xenografts, allografts, and alloplasts. Ideally, the material for bone regeneration should be able to form a new bone, and the formation of the new bone should balance with resorption [36].

The first biomaterials used for grafting areas with bone deficiencies were autografts. Autogenous bone is considered the gold standard for grafting biomaterials for its three main properties: osteogenesis, osteoinduction, and osteoconduction. Advantages of autogenous bone grafts include early vascularization, osteoinductive properties, low cost, and minimal morbidity. Recent research on cortical bone chips revealed that the paracrine effect of bone chips has a significant impact on bone regeneration. Autogenous bone can be harvested near

the receiving site to reduce morbidity. Using a bone scraper may reduce the treatment time and simplify harvesting of the autogenous bone [37].

Allografts are bone grafts collected for transplantation purposes from one person to another and have widespread use. They are important for the treatment of congenital, traumatic, degenerative, and neoplastic bone defects. The advantages of allografts include availability and reduced morbidity, since harvesting bone from an intraoral site is no longer required. The main disadvantage is the possibility of transmission of infection from the donor to the recipient. Possible transmittable infections include malignant neoplasms, degenerative bone diseases, hepatitis B, hepatitis C, and HIV. Donors are carefully screened, and graft materials are meticulously processed to reduce disease transmission. Allografts are not osteogenic and thus, healthy bone formation takes longer compared to that with autogenous bone grafts. There are two main forms of allografts: mineralized freeze-dried bone allografts (MFDBA) and DFDBA. In FDBA, the graft is dried at low temperatures throughout the entire process. In DFDBA, the mineralized phase of MFDBA is removed so that collagen and BMPs are exposed. If this mineral phase is not removed, the bone induction process is not observed. MFDBA is mainly used for its osteoconductive properties and space maintenance. Cortical bone chips are generally preferred for allografts because of their low antigenic activity and high levels of collagen [36].

Grafts obtained from a donor in a different species are xenografts (also called heterogeneous grafts). Xenografts are composed of deproteinized spongiform bones naturally obtained from other species such as horses or cows. Heterogeneous bone grafts have been proposed to fill bone defects; many clinicians have reported that these grafts have little to no osteogenic potential and may instead be used as scaffolds for space maintenance and long-term bone formation. Bovine bone is the best and most commonly preferred source of xenografts. The risk of transmission of diseases, such as spongiform encephalopathy in cattle, is insignificant due to the grafts deproteinization process. Inorganic and protein-free bones are materials in which only the natural calcium phosphate in the bone is retained. This material consists of unsaturated calcium apatite crystals, and provides long-term low resorption space maintenance, shown to remain 10 years postoperatively. Xenografts inhibit resorption of the grafted site but may negatively impact healing by decreasing the rate at which the implant surface area is integrated with the newly formed bone. Used in cystic cavities, alveolar ridge augmentation, extraction sites for implant placement, and sinus lifting, xenografts are viable materials, when a high osteogenic potential is not imperative. Xenografts can also be mixed with autogenous bone grafts. Such a composite graft material with osteogenic properties can be successfully used for horizontal and vertical ridge augmentations [19].

Alloplastic biomaterials are synthetic graft materials. Biocompatible synthetic graft materials have been used for the last two decades to avoid the disadvantages of allografts and xenografts. Alloplastic materials are not osteoinductive, but they can provide space maintenance and act as a scaffold for new bone formation; this means that they are osteoconductive. Advantages of alloplastic materials include being risk free in terms of cross infection, their availability, being sterilizable, and their biocompatibility. Alloplasts used in augmentations are solid or porous polymers, hydroxyapatite (HA), and calcium triphosphate ceramics, or combinations of these materials [20].

Calcium phosphate ceramics can be both osteoinductive and osteoconductive. Osteoinductivity occurs with the formation of a hydroxyapatite (HA) layer immediately after implantation. Ca2 + and PO4 ions required to form this layer are removed from the bone surrounding the graft. With excellent biocompatibility and without systemic toxicity or foreign body reactions, calcium phosphate ceramics are promising biomaterials that require further clinical investigation. Synthetic hydroxyapatite is one of the most commonly used alloplastic materials because of its chemical composition, which is similar to the human bone. It is nontoxic, has high chemical stability, and causes less inflammation and antigenic reactions. Another important property of HA is that the microstructure can be controlled to induce the formation of pores in the material that permits the migration of new bone tissue and blood vessels. Clinical applications, such as bone defect repair, alveolar ridge preservation after tooth extraction, ridge augmentation, and sinus grafting possibly combined with autogenous bone, are possible with HA [36].

Tricalcium phosphate (TCP) is a biocompatible and bioabsorbable material. However, due to rapid dissolution within 6 weeks, it is not an optimal bone substitute in terms of space maintenance. It is similar to the mineral structure of the bone in terms of its chemical composition and crystal structure. It follows similar healing steps with other graft materials. The known disadvantages of TCP are indicated as unpredictable and rapid resorption rate [19].

6. Membranes

Various types of membranes have been used for tissue regeneration, with the aims of support and maintenance of the treatment area. The barrier membrane allows the migration of regenerative cells within the confinement area, while this technique prevents the migration of undesired cells into the wound area. There are two main groups of membranes: resorbable and nonresorbable.

6.1. Resorbable membranes

Graft materials have been used with resorbable membranes for guided bone regeneration. Ever since resorbable membranes have no stable fixed shape, it is feasible to utilize them for GBR. Resorbable membranes that are developed nowadays are prepared from glycosides and lactic polymers. Absorption of these membranes by hydrolysis takes a minimum of 6 weeks and is completed in exactly 8 months. Traditional resorbable membranes, using polymers like polylactic acid, demonstrated therapeutic problems due to their inflammatory properties and reaction to foreign bodies upon degradation. Due to premature membrane resorption, minimal inflammatory reaction may occur, but clinical observations show that the inflammation does not prevent healing. Resorbable membranes possess qualities such as low possibility of complication, membrane subtraction after healing, reduced morbidity, and easy manipulation. These types of membranes as effective as conventional expanded polytetrafluoroethylene (e-PTFE) in recent experiments [37].

Polymers have had long and widespread use as biomaterials. Resorbable polymers have a remarkable advantage since they do not require a second operation after implant placement.

The body can absorb these materials over time. Polylactic acid membranes can retain their long-term durability. They can be prepared in small sizes and yield more moderate foreign body reactions. Furthermore, slow degradation makes the substance less aggressive. Thus, the surrounding tissue produces less reactions. The clinical use of polylactic acid membranes is that they can serve as barrier materials that can guide the periodontal ligament and bone cells that in turn can be shaped according to the morphology of the defect when manipulation is evaluated. When evaluated in terms of membrane reliability and toxicity, any negative tissue reaction that can be attached to this membrane in surgically created defects does not show any anatomical defects in the regenerated portions [38].

Collagen membranes have recently been preferred due to their biological advantages. They are strong and resistant to deformation and have high-calcium-binding properties. In addition, collagen membranes are biocompatible and are as matrix materials in guided tissue regeneration and with hydroxyapatites. Collagen membranes do not possess immunogenicity; they are well-qualified and have demonstrated excellent long-term clinical outcomes (**Figures 5** and **6**) [39].

Synthetic barriers, such as collagen and PTFE barriers, also yield successful clinical results. They occur in the form of lactic acid and glycolic acid polymers. Although directed tissue regeneration membranes are widely accepted as a treatment modality, their clinical use should be approached with care. These membranes may cause problems such as exposures, risk of bacterial infiltration, and incomplete closure of the operative site. Degradation is usually through hydrolysis when membranes that are resorbed are used. This leads to the formation of an acid cycle, which is a negative effect on bone formation [40].

6.2. Nonresorbable membranes

Reinforced nonabsorbable membranes are used when higher bone augmentation is required. e-PTFE, titanium-reinforced e-PTFE, dense polytetrafluoroethylene (d-PTFE), nano-PTFE, and titanium mesh membranes are known as nonresorbable membranes. Nonresorbable membrane barriers require a second surgical procedure to remove them from the site of augmentation. In large bone defects, the e-PTFE membrane cannot adequately cover the existing

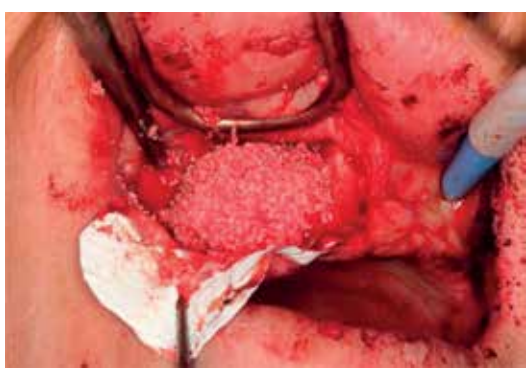

Figure 5. Horizontal augmentation of alveolar ridge, application of xenograft and collagen membrane.

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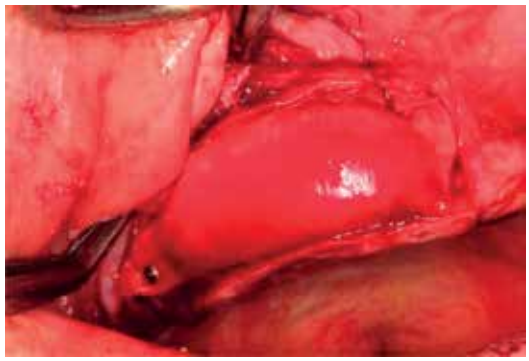

Figure 6. Stabilization of collagen membrane with miniscrews.

space unless supported by graft material. The most important disadvantage is that it requires a second surgical operation because it cannot be resurfaced. It has become preferable to use membranes that are resorbed because of the risk of tissue damage and economic damages to the patients due to a second operation. In addition, nonresorbable e-PTFE membranes are disadvantageous because these membranes involve a high incidence of soft tissue problems, such as exposure, especially when compared to resorbable membranes [41].

Comparison of e-PTFE and resorbed membranes reveals that bone regeneration with e-PTFE membranes is greater, if no exposures occur [40]. Because e-PTFE has no tolerance to exposure, e-PTFE membranes must be completely healed during the primary healing procedure. Currently, because of the complications related to membrane exposure, e-PTFE membranes are not commonly used in GBR treatments. Instead, d-PTFE membranes, which are titanium-reinforced nonresorbable membranes, are used for the reconstruction of critically sized defects. A d-PTFE membrane is used because unlike e-PTFE, d-PTFE continues to be functional even if exposed to the oral cavity. Nano-PTFE membrane is more flexible than e-PTFE; therefore, manipulation and adaptation in this type of membrane is easier. Nano-PTFE has 0, 2–0, and 3 pores. These small pores limit the access of epithelial growth and bacterial infiltration in the augmentation area [41].

The advantage of strengthening membranes with titanium is that it maintains regeneration of the region and obstructs pressure on graft material, soft tissue subsidence, and resorption. Its surface structure and pores are designed to prevent bacterial migration and retention. Soft tissue provides a suitable environment for bone formation and neovascularization in the region by reducing migration to the defect site. They are strained membranes and do not bend but are also resilient enough to prevent perforation of the soft tissue [42].

7. Platelet concentrates

Recently, there has been increasing interest to promote bone formation. Platelet-rich plasma (PRP), growth factors, and BMPs are used to accelerate bone augmentation [43]. Coagulated blood acts as a scaffold for bone formation [44].

7.1. Platelet rich plasma (PRP)

The plasma rich in thrombocytes obtained from autogenous blood tissue is called PRP. PRP contains high proportions of thrombocytes as well as growth factors and other components [45]. PRP is obtained by centrifugation of blood, and 95% of the platelets comprise 4% red blood cells and 1% white blood cells. The most common advantage of PRP is that it accelerates hard and soft tissue healing. PRP can be injected directly into the wound area to accelerate tissue healing or it can be used with graft materials [46].

PRP has a long shelf life, but it should be used quickly. This is because 95% of the growth factors available in PRP are released within 1 h and the activity lasts for 7 days [47].

The use of PRP in oral maxillofacial surgery has been increasing. PRP secreted by growth factors accelerate the healing mechanism of the bone tissue. It has been shown that PRP increases mature bone density by 15–30% [48].

Furthermore, PRP allows a nonspecific immunoreaction to occur. Leukocytes in this context and interleukins secreted from these leukocytes are also activated by the activation of macrophages. Bacteria exhibiting antimicrobial activity of PRP are *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Cryptococcus neoformans* [49].

7.2. Platelet rich fibrin (PRF)

The PRF protocol was developed by Choukroun in 2001. The goal of PRF is to obtain a membrane that is rich in plagioclase-like factors. The acquisition protocol is not dependent on a specialized medical device but can easily be implemented by clinicians. PRF is obtained by removing autogenous venous blood from the dry glass tubes and then centrifuging it at low speed.

Since no anticoagulant is added to the blood in PRF, blood coagulation mechanism begins. PRF has three layers: red blood cell at the bottom, cells plasma at the top, and PRF clot in the middle. This clot is a 3D strong fibrin matrix structure, in which leukocytes and platelets are present in high concentrations [50].

Previous studies have reported the positive clinical and radiographic results for the efficacy of PRF in intrabony and mandibular defects [51].

Platelets help repair damaged tissues by releasing growth factors such as PDGF, TGF-β, VEGF, IGF-1, FGF, and EGF. The granules in the platelets also stimulate cellular growth and proliferation; similarly, chemokines and cytokines are involved in the regulation of tissue regeneration and treatment of inflammation. Platelet granules are important protein sources for the activation of other cells [52].

7.3. Bone morphogenetic protein (BMP)

Recombinant human bone morphogenetic proteins (rhBMP) are used in osteogenic regenation in addition to its use in pulp amputation treatment for new osteodentin formation in the presence of inflammation [53]. It has been reported that the recombinant human proteins

repairs the pulp to form new dentin [54]. However, half of the morphogenesis is achieved due to the limited lifetime of the carrier at very high concentrations [55].

An ideal carrier has not yet been identified, since the cost for this is high. These factors directly influence gene therapy instead of being applied along with morphogenesis, which is a desirable treatment approach [55].

8. Conclusions

This chapter is concerning the dental implant placement. It is one of the most reliable and predictable treatment choices in modern oral surgery. The ways to regenerate the bone to place the implants with the desired dimensions are as follows: (1) guided bone regeneration, (2) socket grafting, (3) allograft bone block grafting, (4) intra- and extraoral autogenous bone block grafting. There are many scaffold biomaterials available that are used as templates for new bone formation. In recent years, biomaterial usage for the reconstruction of hard tissue defects has dramatically increased. Combination of scaffold biomaterials with growth factors presents promising results. In the future, there is no doubt that autologous bone usage will be replaced with artificial tissue engineering.

Conflict of interest

There is no conflict of interest in this study.

Author details

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Section 6

Tissue Regeneration for Tendons

Chapter 10

Tissue Engineering of Tendons

Johanna Buschmann

Additional information is available at the end of the chapter

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Abstract

Critical size tendon defects demand for tissue samples replacing the missing tissue and guiding an effective healing. Autografts, allografts, or xenografts represent viable options; however, limited availability and donor site morbidity go along with this approach, representing big disadvantages. Tissue engineering of tendon tissue is a further strategy fulfilling this need. Basically, an appropriate scaffold material is developed and tested for its biomechanical suitability as a graft material. In addition, cell seeding might improve biointegration of the tissue engineered construct (TEC). Different cell sources as well as different cultivation procedures can be applied in order to tune the envisioned primary strength of the TEC. In this chapter, *in vitro* fabrication protocols and mechanical tests as well as animal *in vivo* experiments will be presented—covering various (bio)materials, cell types, and cultivation procedures.

Keywords: tendon, graft, scaffold, biomechanics, gene therapy, growth factor

1. Introduction

Tendon injuries as encountered by accidents may end up in complete ruptures, going along with tissue defects that have to be replaced with the aim to regain full function—without pain. In order to offer the body suitable substitutes for what it has lost, materials are needed that guide and stimulate the healing process and finally lead to a fully integrated and sufficiently stable tissue. Main problems occurring after tendon rupture repairs are insufficient strength (leading probably to re-ruptures) and adhesion formation (leading to a diminished range of motion) [1].

Best grafts for the reconstruction of injured tendons are obviously tendons themselves, however, although sometimes possible, tendon grafts are very limited in terms of availability and have to be decellularized before application if they are allografts or even xenografts to avoid

transplant rejections. Only autografts are easily transplanted—but the donor site morbidity may cause a lot of pain and go along with impaired function. In addition, other disadvantages of autografts are reported to be insufficient strength [2] because other tendons than the one to be replaced might be different in strength, cellularity as well as gliding capacity [3].

Hence, an excellent alternative to decellularized tendons is the tissue engineered construct (TEC) aimed to be attached to tendon stumps [4] (**Figure 1**). In this field, tissue engineering has covered natural materials like collagen constructs [5], combinations of natural and synthetic components as realized in PLGA and alginate [6] or entirely synthetic polymers such as PCL ± PEO [7]. Many reports on seeding cells onto the corresponding materials have determined their impact, including extracellular matrix deposition and inherently going along changes in stability [8]. Other strategies include growth factors implemented in the graft material [9, 10] with the ultimate aim to be released sustainably to the repair site in order to support and accelerate the innate healing process [11].

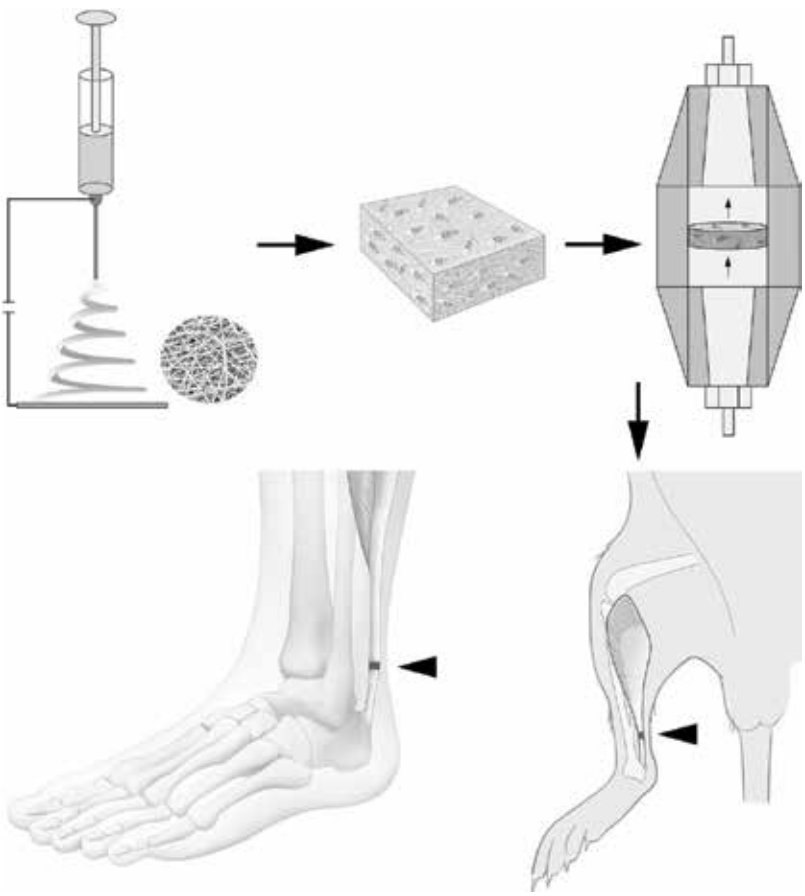

Figure 1. Fabrication of a tissue engineered construct. As a first step, a scaffold material is used and processed as exemplified by electrospinning. Then, cells may be seeded onto the construct. After that, the cell-seeded construct may be cultivated under static conditions or under perfusion in a bioreactor before being implanted into an appropriate animal model. As a final step, performance of the TEC is assessed in a clinical trial.

In this chapter, natural and synthetic materials as well as combinations of them are presented. Moreover, different types of cells seeded onto TECs are compared and their performance *in vitro* and *in vivo* [12] are discussed in a step-by-step manner with criteria set as evaluation milestones [13]. Although many of these approaches are highly promising in animal studies, they did not yet find their ways into clinical application because the success of new graft materials is finally dictated by clinical outcomes of studies where graft materials are implanted into the human body—and clinical trials are not only expensive, they also take a long time to be performed. Tissue engineered constructs that were developed 10 years ago might only now be ready to be judged and tested in terms of clinical success or failure.

2. Native tendons

2.1. Structure and composition of tendons

Before we turn our interest toward tissue engineering of tendons, a brief summary of what native healthy tendon tissue is composed of and of the characteristics of human and selected animal tendons is given here. The hierarchical structure of the tendon tissue is very well-known and has been characterized by multiple imaging and analysis techniques [14]. Starting with the smallest molecular entity, tropocollagen molecules assemble to form microfibrils. Covalently connected, these microfibrils form sub-fibrils and fibrils of the collagen which is typically seen in histological sections of tendon tissue as slightly waved "crimps" [15, 16]. Fibrils form bundles resulting in fascicles. Between the fascicles, there are cell-rich layers called endotenons that can be very well seen in histological sections, as the tenocytes form "lines," one behind each other connected by gap junctions that are important for mechanotransduction [17–19].

In addition, there are some tendons that have a peritenon around the whole tendon. The peritenon is a thin sheath around the tendon, but should not be confused with the tendon sheath on intrasynovial tendons [20]. More information on the organization of the tendon tissue are found in several articles [14, 21, 22], with a special emphasis on the extracellular matrix (ECM) [23].

In the ECM, the main component is collagen I (around 95% of the dry weight). The non-collagenous part of tendons is composed of proteoglycans like lubricin, decorin or biglycan, glycosaminoglycans (GAGs; typically encountered as chondroitin sulfate, dermatan sulfate or heparan sulfate) and glycoproteins such as fibronectin. Proteoglycans are important for tissue hydration (especially decorin) and it was found that they are essential for limiting the viscoelastic behavior by preventing tissue fatigue [24]. When GAGs of an extracted fascicle were enzymatically digested, they exhibited higher reductions in failure stress and more stress relaxation, supporting the regulation of viscoelasticity [24]. Noteworthy, water makes up 60–80 wt% of the entire tendon tissue and is—together with the GAGs—a highly important component regulating viscoelasticity [25]. Moreover, elastin has not to be neglected although it makes up only 2% of the tendon dry weight. Elastin fibers are found closely to the tenocytes—the cells in the tendon tissue [26].

Tenocytes are the mature tendon cells, while tenoblasts are the immature ones. Tenoblasts build up the ECM components. They are spindle-shaped, very similar to fibroblasts—and their

morphology changes upon aging [27] and mechanical loading [28]. Surface marker characterization of tenocytes and tenoblasts includes tenomodulin [29], which is induced by scleraxis (Scx), a transcription factor identifying tendon cells during development [30]. Other cell types occurring in tendon are synovial cells, typically found in the tendon sheath and synovial lining cells. One subtype of synovial lining cells produces hyaluronic acid, an important lubricant facilitating the gliding of the tendon in the sheath [31]. In addition, tendons do also have stem cells, primarily residing in a niche composed of biglycan and fibromodulin [32]. Like other adult stem cells, tendon stem cells are able to self-renew, form colonies, and differentiate into lineages like osteoblasts, chondrocytes, or adipocytes [33, 34].

2.2. Biomechanical baseline values

For successful tissue engineering of tendons, it is essential to know the basic mechanical properties of the tendons that have to be reconstructed in order to plan processing steps accordingly. Hence, *ex vivo* determined biomechanical properties of target tendons are crucial and should always be taken as background information to compare (a) *in vitro* mechanical properties of TECs and (b) *in vivo* mechanical properties of TECs [13]. Tendon ultimate stress values of all human tendons are in a range of approximately 5–80 MPa. *Supraspinati* of the shoulder exhibit quite weak tendon tissue in the posterior portion with only 4 ± 1 MPa [35], while Achilles tendons have ultimate stresses of 79 ± 22 MPa [36].

The age influences the stability of the tendon tissue; while Achilles tendons of old people aged 79–100 years were reported to have ultimate stresses of 48 ± 16 MPa, younger people (36–50 years old) had corresponding values of 73 ± 8 MPa; interestingly, an age group in between 52 and 67 years had the strongest Achilles tendons with 81 ± 14 MPa [37]. Besides age, also gender plays a significant role when mechanical properties of tendons are assessed and compared; female donors usually have weaker tendons and ligaments than male donors [38], however, weaker and softer Achilles tendons of women compared to men might also be a consequence of different levels of exercise—and therefore cannot only be attributed to gender [39].

Besides these intrinsic factors (age and gender), physical activity also plays an important role and has a major impact on tendon strength and elasticity. As a consequence, surgical intervention at a ruptured tendon of an athlete might need a different graft material compared to a ruptured tendon of a person that does not do any exercise beyond daily low-impact activities. Interestingly, also exercise in elderly people has a massive impact on tendon strength and elasticity. In a study performed with two groups of elderly people [aged 74 ± 5 years (n = 9) for group 1 and group 2 had and age of 68 ± 6 years (n = 8)], significant impact on the stiffness and elastic modulus of the patellar tendon was found when assessed by ultrasound measurements. Group 1 did one lesson of exercise per week going only to 40% of their maximum capacity, while group 2 had two lessons weekly and went to 80% of their highest capacity. As a result 12 weeks later, the elasticity of the tendons in group one was not changed, while group 2 had 1.6-fold increased stiffness and 1.5-fold increased elastic modulus [40]. Hence, intrinsic factors should not be interpreted alone; however, extrinsic factors like exercise and other physical activities should be considered too.

Compared to humans, the animal realm covers a wider range of mechanical properties; from rat tendons to horse tendons, there is a span of one order of magnitude in ultimate stress; with horse *flexor digitorum superficialis* having values of 109 ± 8 MPa [41], while rat Achilles tendons

only have ultimate stresses of 16 ± 6 MPa [42]. In terms of elasticity, the range for animals is also larger than for humans. While for human tendons it is up to around 800 MPa, for animals, values 1.5 times as high are found like for the rabbit *flexor digitorum profundus* which has an elastic modulus of 1166 ± 281 [43] or the horse *flexor digitorum superficialis* with a modulus of 1189 ± 63 MPa [44]. Xenografts, although rejection problems may arise, might nevertheless be useful starting points if refinements by cell seeding or other cues manipulating the graft are applied too. Otherwise, tendon tissue engineering intended at veterinary clinical application should include such baseline values when planning to fabricate appropriate TECs.

3. Natural materials

3.1. Collagen

Tendon tissue basically consists of type I collagen [14, 45]. Therefore, many approaches in tendon tissue engineering take collagen as a material in order to fabricate appropriate TECs [46]. It has to be noted that mechanical properties of collagen greatly depend on the processing. Kumar and co-workers produced robust planar collagen fiber constructs by drying collagen gels to form dense collagen mats that were layered [47]. With this approach, they were able to tune ultimate stress values between 0.6 and 1.8 MPa; if they used an additional crosslinking step, the range of ultimate stress increased to 4.7 up to 10.5 MPa [47]. As for the elastic modulus, not cross-linked collagen mats exhibited elastic moduli of 2.0–6.3 MPa; with crosslinking, however, such fabricated mats had moduli of 52–114 MPa [47]. Obviously, with only one processing step (crosslinking), mechanical characteristics could be changed by an order of magnitude, enabling the tissue engineer to adapt his material to the mechanics envisioned.

Also, commercially available collagen scaffolds show a wide range of mechanical properties and may be chosen upon those selection criteria [48]. Generally, such scaffolds are patches that are used as augmentations in order to increase the primary repair strength after operation. The following commercially available collagen patches are described further in [48]. They are presented with decreasing strength (**Table 1**).

Trade name	Tissue	Ultimate load (N)
GraftJacket® extr. 2.0	Human dermis	229 ± 72
MaxForce® 1. 4	Human dermis	182 ± 50
GraftJacket® 1.0	Human dermis	157 ± 38
Permacol® 1.0	Porcine dermis	128 ± 26
TissueMend® 1.1	Fetal bovine dermis	76 ± 22
TissueMend® 1.2	Fetal bovine dermis	70 ± 13
Restore® 1.0	Porcine small intestinal submucosa	38 ± 3
CuffPatch® 1.0	Porcine small intestinal submucosa	32 ± 4

Table 1. Selected commercially available collagen scaffolds sold as patches for tendon or ligament augmentation in the order of decreasing strength according to [48].

Depending on the processing technique and the final architecture and structure of the collagen scaffold, ultimate stress and elastic modulus vary over a range of six orders of magnitudes, with the following increasing order:

sponges < gels < yarns, mats < cross-linked mats < cross-linked yarns <3D extruded fibers

As shown by Kato and colleagues, extruded collagen fibers highly resemble mechanical characteristics of rat tail tendon tissue [49], with elastic moduli >1000 MPa and ultimate stress >600 MPa. However, these mechanical assessments were made under dry conditions. As tendons are hydrated tissues, wet conditions should rather be taken into account. For that reason, Zeugolis et al. compared extruded collagen fibers under wet and dry conditions and found that wet extruded fibers were swelling (increase in CSA), while ultimate stress values decreased by factors up to 2000 [50]. Therefore, other optimization strategies like blending collagen with PEG (polyethylene glycol) were undertaken in order to achieve not only the desired fiber thickness but also envisioned mechanical properties [51]. Moreover, crosslinking of extruded collagen fibers with different chemical agents like aldehydes and isocyanates, biologically by microbial transglutaminase or physically by photo-oxidation was compared in terms of fiber diameter and mechanical properties [52]. A total of 16 different ways for crosslinking were compared and the high variability in characteristics was summarized [52].

3.2. Silk

Silk is derived from silkworm cocoons named *Bombyx mori* (mulberry silk) consisting of two fibroin proteins, and has been approved by the Food and Drug Administration [53]. The physical properties of silk fibroin (which is achieved after sericin is removed) are ideal for tendon grafts. Moreover, silk fibroin is biodegradable and compatible and can also be structurally changed and adapted for different purposes [54]. Silk fibroin exhibits ultimate stress values up to 4800 MPa, which is far beyond maximum ultimate stress limits of human tendons (approximately 80 MPa) and animal tendons (around 120 MPa). Physical properties can be tuned by giving the silk fibroin different architectures. Li and Snedeker showed that wired, braided, and straight silk fibroin fibers behaved differently in biomechanical fatigue tests [55]. They found that a wired structure best fitted their final target which was an anterior cruciate ligament. In addition, also knitted silk fibroin gained from a non-mulberry silk intended at tendon tissue engineering has been tested *in vitro*, and Musson and co-workers found that cell attachment and growth was satisfactory [56]. Finally, biphasic silk fibroin scaffolds with different pore alignments (anisotropic and isotropic) mimicking the tendon-bone interface are very promising TECs based on this natural material [57].

Often, silk is combined with other materials like collagen [58, 59], PDLLA [60], or PLGA [61, 62] in order to manipulate and adapt the TEC under view. As a promising example, silk fibroin was combined with PCL and electrospun nanofibers of this blend were seeded with rabbit dermal fibroblasts, with the result that silk fibroin favored and supported cell proliferation compared to blank PCL and tendon-specific proteins like collagen and tenascin-C were increased and deposited to a higher amount in an *in vivo* experiment using New Zealand White rabbits and an Achilles tendon partial defect [63]. Moreover, also biomechanics were

considerably enhanced by the presence of silk fibroin in this blend compared to mere PCL [63] because silk is a very stress-resistant material and can be tuned so well in order to cover a wide range of mechanical properties; it has not only been considered for tissue engineering of tendons but also for applications in bone tissue engineering [64–67].

3.3. Chitosan

Chitin and its derivative chitosan are getting more and more attractive as a suitable natural biomaterial for tissue engineering purposes [68], especially for tendons [69]. In a combination with poly acrylic acid, composite films were fabricated by a layer-by-layer technique. These films exhibited elastic moduli of 27–420 kPa suitable for tissue engineering of tendons exhibiting low elastic moduli [70]. Other composites like chitosan-hyaluronic acid were used to close defects of *infraspinatus* in a rabbit model. The result was that ultimate stress and elastic modulus were significantly increased as compared to defects closed without this scaffold [71]. Moreover, the same composite material was also used for *medial collateral ligament* reconstruction in a rabbit model and it was found to be a promising substitute in case cells were seeded on the chitosan-hyaluronan [72].

Chitosan in combination with collagen has also been investigated to serve as a material for tissue engineering: addition of chitosan to bovine and salmon collagen scaffolds improved the mechanical properties by increasing the compressive strength and the swelling ratio [73]. Moreover, a rat Achilles tendon study, where a scaffold based on chitosan-β-glycerophosphate-collagen was used, demonstrated the effectiveness of this composite material for this purpose [74].

4. Synthetic materials

If synthetic polymers are used for (tendon) tissue engineering, the fabrication process highly decides upon its biocompatibility and its effectiveness as graft. As nicely shown by Prof. Ratner, the same polymer, once applied as a porous foam and once as a dense block, can evoke quite different reactions of the body: while the porous material is penetrated by ingrowing cells as well as vasculature and there is practically no foreign body reaction, the dense block is encapsulated as a foreign body going along with an inflammation reaction [75]—*in vivo veritas* [76]. Hence, the processing of a synthetic material, mostly polymers in tendon tissue engineering, has to be optimized in order to get a biocompatible material that fulfills the requirements encountered in tendon tissue engineering.

Many polymers have been synthesized and modified in order to get suitable materials in terms of implants for tendon repair and regeneration; polyglycolic acid (PGA) [75, 77], polylactic-co-glycolic acid (PLGA) [78], PLGA/alginate composite [6], polylactic acid (PLA) [79–81], poly-L-lactic acid (PLLA) [82–84], polycaprolactone (PCL) [85], polycaprolactone/polyethylene oxide (PCL/PEO) [7], polyurethane (PU) [86, 87], polyethylene terephthalate (PET) [88, 89], DegraPol® [90, 91] (**Figure 2**), nanocarbon fiber [92], and polyurea [93], among others. The architecture of the synthetic materials has to be chosen carefully, as gene expression of (stem) cells may be significantly influenced by the microenvironment that the cells encounter [94].

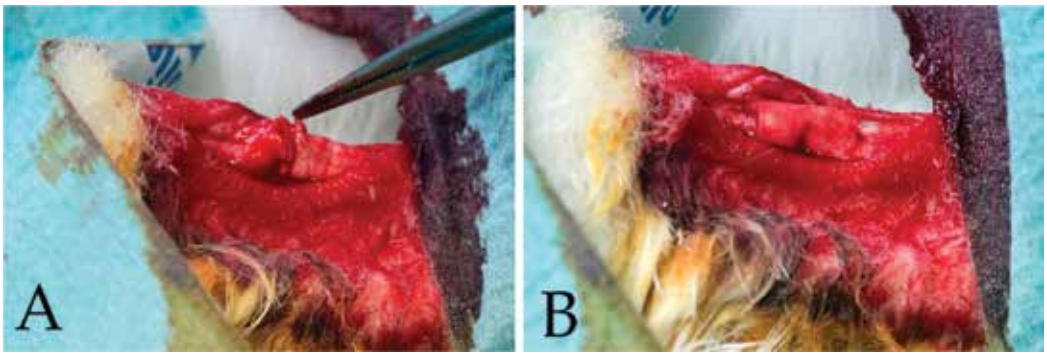

Figure 2. Application of DegraPol®. An electrospun DegraPol® tube is placed around a fully transected rabbit Achilles tendon (A) in order to deliver a growth factor to the repair site. The laceration is sutured by a 4-strand Becker suture and the tube is pulled over (B).

5. Cellular approaches

The potential of stem cells for regenerative medicine and for tissue engineering applications has been reported many times with convincing evidence *in vitro* and *in vivo* and comprehensive information given in recent review articles [12, 95]. Hence, although tenocytes would be the first and self-evident cell source to be used in tendon tissue engineering [20, 96, 97], there are more reports based on stem cells for the same purpose. For example, adipose-derived stem cells were seeded onto biphasic silk scaffold in order to fabricate a tendon-to-bone interface, mimicking the gradient-like structure of the enthesis [57]. Adipose-derived stem cells are easily harvested and differentiated toward a desired lineage [98] and amounts per gram of tissue are higher as compared to other stem cells sources like bone marrow [99]. Hence, these cells are very well suited for tissue engineering purposes, like tendon tissue engineering [100, 101]. Among different stem cell sources, however, the best source of stem cells for tendon tissue engineering is reported to be tendon stem cells, although their availability is limited and the harvesting protocol everything else than easy [32]. An interesting study reports the beneficial effect of seeding tendon-derived stem cells onto a chitosan-β-glycerophosphate-collagen hydrogel scaffold intended to repair an Achilles tendon defect in a rat model [74]. The healing was enhanced as indicated by the improvement in histological and immunohistochemical outcomes. In addition, the increase in the biomechanical properties of the regenerated tissue at both 4 and 6 weeks post-operation also supported the effectiveness of tendon-derived stem cells [74].

The *in vitro* preparation of cell-based TECs highly determines the mechanical properties; cell-seeded scaffolds cultivated under static conditions have different characteristics compared to TECs cultivated under dynamic conditions—as for example cultivation in a bioreactor with medium perfusion flow and/or tensile stretching/compression regimen [102]. Collagen sponges seeded with MSCs have been reported to have significantly higher mechanical properties when cultivated with mechanical stimulation than under static conditions [103]. Also, the expression of collagen I and III are increased upon mechanical stimulation, as shown for rabbit MSC/collagen sponges and murine MSC/collagen sponges [104]. In such approaches of

dynamic cell culture and cell-seeded TECs, physical experimental parameters like frequency, amplitude, medium flow rate, etc., have to be carefully tuned in order to get the desired differentiation (if stem cell-based) and the intended biomechanics [105–108].

Other concepts in tendon tissue engineering are based on decellularization of a natural xenograft in order to avoid immunorejection [4, 109–111] or decellularization of a primarily cell-seeded construct in order to generate a scaffold coated with the components of the ECM of a certain cell type (instructive ECM) cultivated under well-defined conditions [112, 113]. Such decellularized graft materials can be applied in daily clinical practice more easily than cell-seeded TECs; because storage is facilitated without (living) cells [114].

Furthermore, there are gene therapy strategies including adeno-associated viral type 2 vector (AAV2) and micro-RNA related gene therapy aiming at improving strength of the repaired tendon as well as decreasing adhesion formation to the surrounding tissue [115, 116]. Moreover, some approaches deal with delivering certain (growth) factors, supporting the regeneration process of tendons [117, 118].

TGF-β1 plays an important role during tendon healing and has an influence on adhesion formation, an unwanted side effect during tendon healing. Therefore, regulation of TGF-β1 through application of micro-RNA specifically inhibiting the function of TGF-β1 was tested in a chicken flexor tendon model [119]; TGF-β protein expression in the tendons decreased on increasing the vector dosage. As a consequence, the adhesion extent significantly decreased 6 and 8 weeks post-injury; however, tendon strength unfortunately was also reduced [119]. Another study showed that gene therapy to produce supernormal amounts of bFGF or VEGF supported the intrinsic tendon healing in a chicken flexor tendon model—with a significantly higher tendon strength by 68–91% from week 2 after AAV2-bFGF treatment and by 82–210% from week 3 after AAV2-VEGF compared to controls [120]. At the same time, adhesion formation was not adversely affected.

Because decorin and IL-10 downregulate TGF-β1, another approach included co-delivery of decorin and IL-10 transgenes from a collagen hydrogel system to a tenocyte culture *in vitro*. As expected, TGF-β1 was downregulated and simultaneously also collagen I and III and fibronectin. The authors concluded that this approach might be a useful tool against scar formation (extensive fibrosis), the system has not yet been tested *in vivo*, however [121]. Moreover, another AAV-based approach was the delivery of VEGF to chicken flexor tendons; after complete transection of these tendons, 2×10^9 particles of AAV2-VEGF or saline (as control) were injected before they were surgically repaired [122]. The outcome was a significantly increased ultimate strength 4, 6 and 8 weeks post-operation, while the adhesion was unaffected [122]. Hence, such gene therapy approaches might get more significance also in daily clinical life, as they are easily performed (injection of a small volume only) and show promising effects.

Another nice example has been shown using Scx-transduced tendon-derived stem cells in a rat unilateral patellar tendon window injury model. For transplantation, a TEC based on fibrin and transduced cells was used. Tendon repair was significantly improved in terms of histology and biomechanics *in vivo*. *In vitro* results showed that Scx-transduced tendon-derived stem cells expressed tendon- and also cartilage-related genes to a higher level; as for

osteogenic markers (which might be regarded as an unwanted differentiation), the results remained inconclusive [123]. Further reports on gene therapy dealing with the tendon-to-gone interface also used BMPs [124].

6. Drugs to stimulate the healing

Growth factors or drugs aiming at the support of the healing process can be injected directly to the wound site. However, one major problem encountered by doing that is that activity of such biological molecules (often proteins) is short-lived. Hence, a suitable strategy is to take a delivery device realized by an implant material or a TEC allowing sustained release of the drug to the repair site over longer periods of time. Among many others, one interesting growth factor supporting tendon rupture repair is PDGF-BB [125]. It is not only mitogenic but also angiogenic and chemotactic—ending up in accelerated cell proliferation, migration, increased collagen synthesis and vascularity, and finally improved biomechanics of the repaired tendon [125]. Hence, a random-fiber electrospun delivery device in form of a tube was developed which not only allowed the controlled release of PDGF-BB [11], but also acted as an effective physical anti-adhesion barrier [126]. Without the growth factor included, this TEC neither evokes any adverse cellular effects nor influences inflammation reaction toward the implant [90, 91]. With the growth factor, biomechanics improved significantly, underlying promising perspectives for this bioactive implant.

Another approach using tendon-derived progenitor/stem cells seeded onto an aligned random-fiber mesh made of PLLA discovered that tenogenesis of these stem cells was not triggered by the aligned fibers, which was previously hypothesized. Because the expression of histone deacetylases was found to be reduced in the progenitor cells seeded on the aligned fibers, a small molecule (Trichostatin A), which is an inhibitor of histone deacetylases, was incorporated in the aligned fiber mesh. As a result of this bioactive mesh, the corresponding progenitor cells seeded on this TEC showed better tenogenesis and when implanted in a rat Achilles tendon model, the healing was accelerated and improved compared to non-Trichostatin TECs [83].

7. Conclusion

Tissue engineering of tendon substitutes and grafts is a viable option to close critical size defects. The choice of a suitable scaffold material, natural or synthetic, is a decision which should be based on biomechanical baseline values of native tendon tissues and which will direct/affect biocompatibility, cell attachment, or incorporation of factors that support the healing process. Cell seeding and cultivation may be performed under static conditions as well as in dynamic systems using bioreactors. Bioreactors offer perfusion flow resulting in shear stress; additional mechanical stimulation by stretching the cell-seeded TEC may help to improve the mechanical characteristics of the TEC and trigger the desired differentiation if stem cells are involved. Growth factors incorporated in TECs may also support the healing process of the lacerated tendon tissue.

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Cell VI Abilities

Chapter 11

3D Bioprinting: Surviving under Pressure

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Additional information is available at the end of the chapter

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Abstract

Because 3D bioprinting using microextrusion was reported to yield cells with low viability (~40%) after pneumatic pressure (40 psi) printing through stainless steel nozzles, or blunt-end needles, with about 150 μm diameters (28 and 30G), we set out to improve the viability by coating the interior of the nozzles with silicone. For these studies, H9 human lymphoma cells were used to simulate human stem cells in suspension, and cell viability was measured using propidium iodide dye exclusion and flow cytometry. We tried to improve the viability by coating the inside of the 28 and 30G nozzles (1″ length) with silicone to protect the cell membranes from being damaged by the imperfections in the stainless steel nozzle. However, we discovered silicone coating had little effect on viability because imperfections in the nozzle were not the problem. Instead, the cells being placed in hypotonic 3% (w/v) alginate prepared in water prior to printing caused significant cell death (~25%) and considerably more (≥50%) after simulated printing under pressure. By preparing the alginate in isotonic solutions of either phosphate buffered saline or complete culture media, we could use pressures over five times (>220 psi) what most printing procedures use and obtain ~80% viability.

Keywords: force, hydrogel, hypotonic, isotonic, microextrusion, viability

1. Introduction

3D bioprinting is the wave of the future for constructing viable, functional, and biocompatible human organs that will be created from the patient's own stem cells so that antirejection drugs will not be needed after transplantation. Currently, there are three main 3D bioprinting methods: inkjet or "drop on demand," laser-assisted, and microextrusion [1].

Inkjet printing uses thermal or acoustic (piezoelectric) forces to create and eject droplets. Thermal inkjet bioprinting yields good cell viability (>85%), and although localized heating of 200–300°C occurs and the temperature at the head only rises 4–10°C for short durations

(~2 μs), cells can be heat shocked. The heat-shock protein chaperones can protect cells from dying [2] so that they have the potential of passing along DNA mutations that could ultimately result in a cancerous cell. Only a couple of degrees rise in temperature ≥2°C (~39°C) is needed to induce some heat-shock proteins [3]. Acoustic inkjet bioprinting applies voltage across polycrystalline piezoelectric ceramics to induce a rapid change in their shape that creates the pressure to eject droplets. However, the 15–25 kHz frequencies shock the cells: causing membrane damage and cellular lysis [4] and allowing molecules up to 40,000 Daltons (90 Å) in size to enter or exit the cell [5].

Laser-assisted bioprinting yields high cell viability (>95%) [6] and is nozzle-free so that the problem of clogging with materials or cells that other printing methods have is circumvented. In addition, it has microscale resolution of a single cell per drop. The drawbacks to laser-assisted bioprinting are low flow rates due to the high resolution requiring rapid gelation kinetics [7], time-consuming preparation of the ribbons used for printing, metal residues from vaporization of the metallic laser-absorbing layer during printing (nanoparticles), the complexity of making ribbons to print multiple cell types, and the high cost; more germane, it is not clear if this technology can be scaled up for larger tissue sizes other than skin [8], let alone organs.

Finally, microextrusion is the most common and affordable 3D bioprinting method that uses either pneumatic (air) or mechanical (screw or piston) forces to create pressurized dispensing systems [9]. However, this 3D bioprinting method is reported to yield the lowest cell viability of all three methods (40–80%) [10]. In previous studies, this low cell viability was completely attributed to the biofabrication mechanical forces or high pressures applied to the cells [11] rather than to structural imperfections inside the stainless steel nozzles creating membrane damage.

We reasoned if some of this membrane damage was actually due to the imperfections inside the stainless steel nozzle, then we might be able to improve the viability by coating their interiors with silicone. By minimizing these interior structural flaws, we would increase the viability during printing under pressure. However, in the course of our investigation, we found that neither the biofabrication of mechanical forces nor the structural flaws inside the nozzle were causing the reduction in viability but rather it was the hypotonic solution the cells were placed in when the alginate was prepared in water; the cells were placed directly into that hypotonic solution without first adjusting it with salt to be isotonic. This lysed many cells (~25%) until evidently the solution became isotonic from the released intracellular salts leaving the remaining cell population "bloated" or swollen and very sensitive to mechanical forces.

2. Materials and methods

2.1. Chemical formulations

The alginate solution was prepared using medium viscosity sodium alginate (Sigma, St. Louis, MO) dissolved in high-performance liquid chromatography grade water (Sigma Aldrich, St. Louis, MO) to make a 3% (w/v) solution as described previously [10, 11]. We also prepared 3% (w/v) alginate solutions in Dulbecco's phosphate buffered saline (PBS) without magnesium or calcium (GIBCO, Gaithersburg, MD) and in complete culture

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media (see below) with the addition of 1 mM ethylene diamine tetra acetic acid (EDTA; Sigma Aldrich, St. Louis, MO). The 1 mM EDTA was added to crosslink the 2 mM calcium present in the media so the alginate would not solidify. Note that this solution of alginate in complete culture media with EDTA did not solidify for over a year. We sterilized these solutions using either a 0.45 μm syringe filtration system (Nalge Nunc International Corporation, Rochester, NY) with the cell dispensing device described below (**Figure 1**) at the highest force (20 lbs) overnight or a 0.45 μm filtration unit with vacuum suction overnight. Additionally, we tested the viability after passing cells through the syringe and blunt-end needles using this force (20 lbs) in complete culture media or PBS (after washing the cells three times to remove any bound proteins that might afford membrane protection).

Figure 1. The KD scientific model 100 series screw-driven pressure pump with maximum force of 20 lbs is shown housed in a homemade holder attached to a vertical stand with a heavy base equipped with a 3-mL syringe and a 28G nozzle of 1″ in length that was used for the experiments shown in **Figures 2** and **4** (**Figure 3** has the 30G nozzle but same device).

2.2. Cell culture

Human lymphoma H9 cells (ATTC, Manassas, VA) were cultured and maintained in the incubator at 37°C using complete culture medium: CO_2-independent media (GIBCO, Gaithersburg, MD) supplemented with 10% (v/v) heat inactivated, mycoplasma-tested, and endotoxin-free, fetal bovine serum (GIBCO, Gaithersburg, MD), 4 mM glutamine (GIBCO, Gaithersburg, MD), antibiotic (10,000 IU penicillin and 10,000 μg/mL streptomycin), and antimycotic (25 μg/mL amphotericin B) solution (Sigma, St. Louis, MO). We use CO_2-independent media so the cells do not undergo pH shock while being manipulated during or after experiments. This enabled us to leave the cells in culture tubes at 37°C using a constant temperature controlled heating block, Hema-Bath Block Module Heater Type 12,200 Dribath (Baxter Scientific Products, Deerfield, IL), under sterile conditions in the biosafety cabinet until monitored on the first day (0 and 4 or 6 h postexposure). For the 24-h time point, we diluted the samples 1:1 with CO_2-independent complete culture media and maintained the cells in 5 mL sterile culture tubes at 37°C in the heating block or in the incubator (results not shown). These suspension cells were grown and maintained below 1×10^6 cells/mL in culture and were usually used between 4 and 8×10^5 cells/mL for experiments with viability ≥90% as determined by dye exclusion of propidium iodide (PI) using flow cytometry.

Cells were centrifuged in 50 mL centrifuge tubes (Corning, Tewksbury, MA) at 300 × *g* for 7 min, and the media were aspirated to leave cell pellets that were loosened by quickly (2–3 s) vortexing at low speed. These cell pellets were very gently and briefly mixed in the viscous 3 mL of 3% (w/v) alginate solutions with a Pasteur pipet by swirling and slowly pipetting up and down three times to homogeneously disperse the cells as previously described [10]. The cell density was ~3×10^6 cells, as determined by hemocytometer readings, before mixing into 3 mL of alginate to give ~1×10^6 cells/mL.

2.3. Procedure for simulated microextrusion pressure printing

Cells were grown to 4–8×10^5 cells/mL in complete culture media whereby 100 mL was centrifuged at 300 × *g* for 7 min, media aspirated, and then the cell pellet was vortexed and suspended in one of the three, 3% (w/v) alginate solutions (H_2O, PBS, or complete culture media) or suspended in solutions of either PBS or complete culture media to a final density of ~1×10^6 cells/mL or 4–8×10^5 cells/mL, respectively. Different concentrations were used to know if high cell density afforded protection for the cells. For the PBS and complete culture media comparison studies, we washed the cells three times with either PBS or complete culture media, respectively, prior to microextrusion. For the T = 0 time point (actual time < 10 min), we simply collected the cells in a test tube containing 0.25–0.5 mL of complete culture media at room temperature. For the longer daily time points of 4 or 6 h, we put the test tubes at 37°C in a dry-block incubator under the biosafety cabinet. For overnight studies at 24 h, we further diluted the cells 1:1 with complete culture media and put them in tightly capped sterile 5 mL polypropylene culture tubes or T-25 flasks (Corning, Tewksbury, MA).

2.4. Microextrusion cell dispensing system

We employed a mechanical device that uses a screw to create a force with subsequent pressure dependent on the radius of the syringe. The KD Scientific Model 100 series (Harvard Apparatus, Holliston, MA) screw-driven pressure pump with maximum force of 20 lbs was housed in a homemade holder attached to a vertical stand with a heavy base (see **Figure 1**). We used syringes of various sizes (3–60 mL) with Hamilton blunt-end 28G or 30G needles (Harvard Apparatus, Holliston, MA) of different lengths: ½″, 1″, and 2″. These blunt-end needles are referred to as nozzles.

The experiments were performed using various pressures "P" that were calculated in pounds per square inch (psi) using Eq. (1):

$$P = \frac{F}{A} \tag{1}$$

where "F" is the applied force (maximum of 20 lbs was used) and "A" is the area (in inches squared) of the applied force. We performed various experiments using syringes of different sizes, 3, 10, 30, and 60 mL, having radii of 0.17 (8.59 mm), 0.285 (14.48 mm), 0.425 (21.59 mm), and 0.524 inches (26.6 mm), with areas of 0.09, 0.255, 0.568, 0.86 inches^2, yielding pressures of 220, 78, 35, and 23 psi, respectively. Pressure experiments were conducted at room temperature, and the cells were placed at 37°C after treatment until analyzed.

2.5. Chemical coating nozzles

In order to help minimize membrane damage incurred during pressurized simulated microextrusion printing, presumably from imperfections in the stainless steel, we coated the interior of the 28 and 30G blunt-end needles for 5–15 min at room temperature using ~10% (w/v) high molecular weight (500,000 g/mole) polydimethylsiloxane, trimethylsiloxy terminated (Gelest, Inc., Morrisville, PA) in high-performance liquid chromatography grade hexane (Sigma Aldrich, St. Louis, MO). To sterilize the blunt-end needles, we luer-locked them on the syringe, submerged them in 70% ethanol, and then used three 0.5 mL interior washes of 70% ethanol followed by three 0.5 mL interior washes of sterile 0.9% saline (isotonic).

2.6. Cell viability

Cell viability was assessed by the dye exclusion method using PI (Molecular Probes, Eugene, OR) at a final concentration of 1 μg/mL using a slightly modified procedure [12]. Briefly, the PI was added directly to the samples so as not to lose any of the representative cell populations or to create false positives by centrifuging and disrupting the membranes of partially damaged cells. Live cells completely exclude PI, while dead cells allow it to almost instantly pass through their membranes. Cell viability was quantified as percentages using flow cytometry.

2.7. Flow cytometry

The viability of 10,000 cells from each sample was determined by a FACSCanto II (Becton Dickinson, CA, USA) triple beam flow cytometer at medium flow rate in the PerCP-Cy5-5-A (equivalent to FL-2) channel using PI dye exclusion [12]. We gated on the single-cell population. To set the marker for the live cell population, we used cultured cells from the incubator (≥90%) and created the marker for the dead cell population (with viability <10%) using 10 mL of 4–8 × 10^5 cells/mL exposed overnight to a final concentration of 300 μM silver nitrate (unpublished results). The sham-exposed cells underwent the same treatment as the exposed cells except they were not put under pressure through the syringes and nozzles; the cells were carefully mixed in alginate or other solutions and then gently placed into the syringe and allowed to slowly drip out as small droplets.

For each experiment, the live and dead cell controls were used to set the gates for the live and dead cell populations in the PerCP-Cy5-5-A fluorescent channel. We used forward scatter characteristics (FSC-W and FSC-H) to distinguish between single and multiple, or clumped, cell populations. The gate was set on the single-cell population to collect 10,000 cells for further analysis in the PerCP-Cy5-5-A fluorescent channel.

2.8. Statistical analysis

The data in the text and figures are presented as the mean (n = 3) ± standard deviation (SD) computed using the Student's t-test for two samples assuming unequal variance and consider $p < 0.05$ to be significant.

3. Results

For these experiments, we used the pressure pump aligned vertically in a homemade holder attached to a stand with a heavy base at a maximum force of 20 lbs (see **Figure 1**).

After preliminary testing, we realized the hypotonic alginate solution was causing the decrease in viability, so we compared alginate prepared in three different solutions: H_2O, PBS, and complete culture media (as described in Section 2). **Figure 2** shows a very significant drop in viability compared to the control (95 ± 1%) when the cells were placed in alginate prepared in H_2O (Sham, 75 ± 11.6%, p = 0.048; 28G no coat, 21.8 ± 13.5%, p = 0.0056; 28G coat, 14 ± 1.3%, $p = 6.3 \times 10^{-8}$), while the cells placed in alginate prepared in either PBS (Sham, 94.3 ± 0.26, p = 0.16; 28G no coat, 92.9 ± 0.6%, p = 0.025; 28G coat, 87.8 ± 2.1%, p = 0.0062) or the complete culture media (Sham, 96.5 ± 0.4, p = 0.063; 28G no coat, 86.3 ± 2.8%, p = 0.0075; 28G coat, 85.5 ± 1.6, p = 0.00045) although sometimes significantly lower did not cause more than a 10% drop in cell viability using the same conditions: 3 mL syringe, 20 lbs of force giving 220 psi, and 28G nozzles of 1″ length. We show the data for 28G nozzles of 1″ length in **Figure 2**, but note that the ½ and 2″ lengths also did not cause a decrease of more than 10% in cell viability if the cells were placed in 3% (w/v) alginate prepared in PBS or complete culture media. Note that coating the nozzles did not improve the cell viability compared to those that were uncoated. The results for T = 0 are shown because the later time points did not yield more than a 10%

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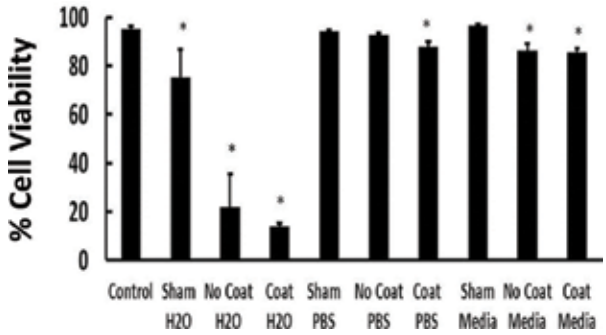

Figure 2. The viability of H9 cells immediately (T = 0 h) following direct cell writing biofabrication using the homemade microextrusion device shown in **Figure 1** equipped with a 3-mL syringe and 28G nozzle of 1″ length using maximum force of 20 lbs to produce 220 psi. Concentrated H9 cells were mixed with 3 mL of 3% (w/v) alginate prepared in different solutions: H_2O, PBS, and complete culture media with 1 mM EDTA. The "no coat" refers to the untreated nozzles and the "coat" refers to the nozzles coated with silicone as described in Section 2.The asterisks indicate significant differences from the controls.

decrease in viability, in agreement with what others found [10], and after 24 h, some of the surviving cells divided increasing the apparent viability.

As shown in **Figure 2**, the nozzle surface chemistry did not affect cell viability, but the hypotonic solution did, so we did not compare coated with uncoated nozzles in **Figure 3**. We wanted to know how a lower gauge nozzle with a smaller diameter would affect cell viability, so we used a 30G nozzle of 1″ length and placed the cells in 3% (w/v) alginate solutions as described previously: H_2O, PBS, and complete culture media with 1 mM EDTA. Using the same conditions employed for the 28G nozzle of 1″ length (i.e., 3 mL syringe and maximum force of 20 lbs yielding 220 psi), we did not observe more than ~8% decrease in cell viability in the alginate prepared in H_2O (14.1 ± 2.4%; $p = 6.9 \times 10^{-6}$), PBS (86 ± 10%; p = 0.21), or media

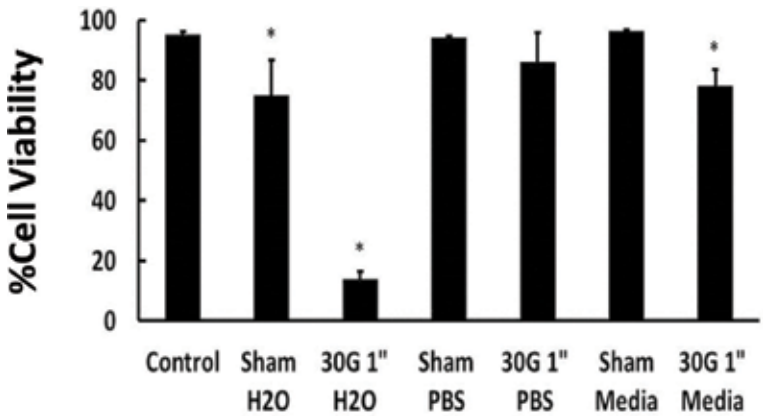

Figure 3. The viability of H9 cells immediately (T = 0 h) following direct cell writing biofabrication using the homemade microextrusion device equipped with a 3-mL syringe and 30G nozzle of 1″ length using maximum force of 20 lbs to produce 220 psi. Concentrated H9 cells were mixed with 3 mL of 3% (w/v) alginate prepared in different solutions: H_2O, PBS, and complete culture media with 1 mM EDTA. The asterisks indicate significant differences from the controls.

(78.3 ± 5.2%; p = 0.016) than after passing them through the 28G nozzle of the same length (**Figure 3**). Note that these differences in cell viability are not statistically significant from those obtained using the 28G nozzles (H_2O p = 0.22; PBS, p = 0.26; media, p = 0.051).

Finally, we wanted to see if we could decrease the cell viability by removing the alginate, as it might be affording protection by coating the cellular membranes. We show the results for 3-mL syringes with 28G nozzles of 1″ length (either coated or with no coat) but found no significant effect on cell viability even at the highest force of 20 lbs resulting in a pressure of 220 psi when in PBS or complete media (**Figure 4**). We also tried 30G nozzles of 1″ length and other syringe sizes, but they only slightly affected cell viability unless the cells were placed in a hypotonic alginate solution.

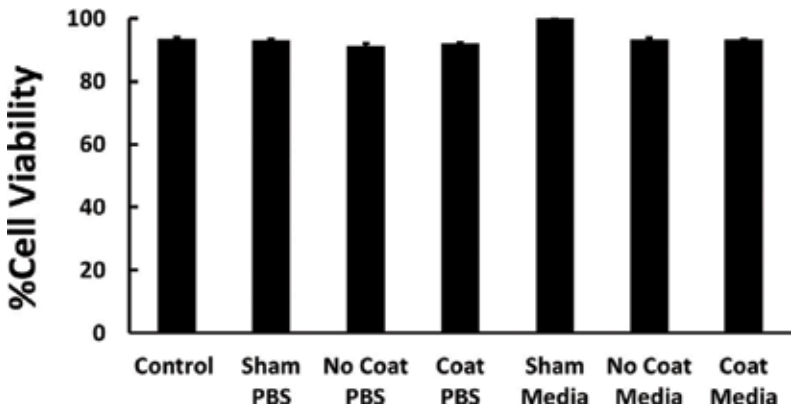

Figure 4. The viability of H9 cells was determined after control, sham, or passing the cells through the 3-mL syringe equipped with a 28G nozzle of 1″ length under 220 psi in either PBS or media in the absence of alginate. The cells were washed with either PBS or complete culture media three times prior to simulated microextrusion printing. The control and shams are as described in Section 2.

4. Discussion

3D bioprinting using microextrusion is the most common and affordable way to print living cells. Microextrusion is a method of direct cell writing that can 3D bioprint using different substances like hydrogels, cell spheroids, and biocompatible polymers facilitating the deposition of multiple cell types with high resolution to accurately fabricate complex structures, like an ear, using computer-aided design software [13]. Among the multitudes of hydrogels, alginate is popular because it is inexpensive and is crosslinked using calcium to give it a solid structural form which can later be reversed using citrate or EDTA. One major advantage to microextrusion printing is the ability to print cells at high densities that are close to physiological conditions, which is needed to construct tissue-engineered organs, maintaining high cell viability using high pressures, and small nozzle sizes are required for fast printing speeds with high resolution. Because microextrusion 3D bioprinting was reported to result in a significant decrease in cell viability yielding between only 40 and 80% live cells in alginate

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solution [10, 11], we decided to try and improve the cell viability by coating the nozzles with silicone in order to prevent membrane damage from nozzle imperfections and high pressures, as the latter was the proposed reason for the decrease in viability [14]. However, we discovered that neither the high pressures nor the membrane damage caused by nozzle imperfections was the reasons for the low cell viability; we found the low cell viability was really caused by preparing the hydrogel (alginate) solution in H_2O, which is a hypotonic solution that causes cell lysis and bloating (swelling). Some studies that reported low cell viability using microextrusion and alginate solutions did not state what solvent the hydrogel was dissolved in, but the fact that low cell viability was observed after printing using increasing pressures suggests a hypotonic solution was the culprit. This appears to be a recurring problem in this field because numerous scientists cite these findings and reproduce them using the same procedure.

During our investigation, we used a variety of syringes (3, 5, 10, and 60 mL) and nozzles (blunt-end needles; 28G with ½″, 1″, and 2″ lengths, and 30G with ½ and 1″ lengths) with only PBS or complete culture media containing H9 cells, but there was no effect on cell viability using the highest force (20 lbs) and 3 mL syringe to yield the smallest area (0.09 inches^2) for the highest pressure of ~220 psi, which is over five times the pressure (~40 psi) that is usually used and is over twice the pressure (~100 psi) most printers can accurately print. Only with the addition of alginate in H_2O did we see an adverse effect on cell viability; there was no effect when the alginate was prepared in either PBS or complete culture media (**Figures 2** and **3**) or if the cells were placed in PBS or complete culture media without alginate (**Figure 4**). The water created a hypotonic solution because the counter cationic ion, alginate cannot enter the cell like chlorine ions (and sodium anions) can because it is too big (MW 216.12 g/mole), so about 25% of the cells initially lysed to create an isotonic solution and the rest of the cells survived but became "bloated" (swollen) during the process. However, our results suggest the bloating made the remaining cells more sensitive to mechanical pressure and caused the observed pressure-dependent decrease in cell viability. By preparing the alginate solutions in PBS or other isotonic solutions like complete culture media, we demonstrate significantly higher cell viability. We also put the cells in PBS and complete culture media without the potential protection of the alginate to see if they would be killed by the pressure or shear force alone, but we did not see any cell lysis or death using the same system (3 mL syringe, 20 lbs, 220 psi) and 28G nozzles of 1″ length (**Figure 4**). In addition, we did not see any significant decrease in cell viability with ½″ or 2″ long nozzles (results not shown). Furthermore, the 1″ long 30G nozzle also did not cause any appreciable decrease in cell viability (~7% decrease compared to 28G, **Figure 3**).

The results presented here show the low cell viability found during some mircoextrusion 3D bioprinting studies using alginate was due to placing the cells in a hypotonic solution causing cell lysis and bloating that makes the cells more sensitive to mechanical pressure during printing, which has been modeled [14]. Here we show this problem can be easily resolved by using isotonic solutions like PBS or complete culture media (0.9% saline is also suitable). Furthermore, the so-called recovery or increase in cell viability after 24 h [10] can be attributed to the division of the living cells rather than the recovery of membrane or other cellular damage, as noted by the increase in total cell number. The reason there is a decline in viability with increasing pressure or decreasing nozzle diameter can be attributed to the increasing shear forces causing

increasing amounts of cellular damage resulting in increasing cell death via apoptosis [11] (and our unpublished observations) but only when the cells are placed in hypotonic solutions.

5. Conclusions

Microextrusion is an excellent 3D bioprinting method that can yield high cell viabilities (≥85%) similar to Inkjet printing using 28G nozzles of either 1/2″ or 1″ lengths for pressures up to 220 psi as long as the hydrogels or solutions are isotonic. Good viability of over 75% can also be achieved using 30G nozzles of 1″ or shorter lengths using 220 psi. Thus, cells can survive with good viability (~85%) under considerable pressure for short periods of time during the microextrusion 3D bioprinting process if they are in isotonic hydrogels.

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Conflicts of interest

None.

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Abbreviations

EDTA	ethylenediaminetetraacetic acid
PBS	phosphate buffered saline
PI	propidium iodide
psi	pounds per square inch
SD	standard deviation

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Tissue regeneration is a vast subject, with many different important aspects to consider. Regenerative medicine is a new branch of medicine that tries to change the course of chronic diseases and, in many cases, regenerates the organ systems that fail due to age, disease, damage, or genetic defects.

The main purpose of this book is to point out the interest of some important topics of tissue regeneration and the progress in this field as well as the variety of different surgical fields and operations. This book includes 7 sections and 11 chapters that provide an overview of the essentials in tissue regeneration science and their potential applications in surgery.

The authors of each chapter have given consolidated information on ground realities and attempted to provide a comprehensive knowledge of tissue engineering and regeneration. This book will be useful to researchers and students of biological and biomedical sciences (medical and veterinarian researchers).

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