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Cells are the building blocks of tissues and organs. Therefore, cell source is a critical issue for tissue engineering. An ideal cell source should be sufficient in quantity, compatible with the immune system of the recipient and free of pathogens or contamination. Depending on the specific tissue engineering application, the cell source can be autologous, allogeneic or xenogenic. Traditionally, fully differentiated cell types are used to engineer tissues. However, for many cell types, differentiated cells from adult tissues often have little or no proliferation potential.

In the past few years, the advancement of stem cell biology has opened a new avenue for tissue engineering. Stem cells can be isolated from adult tissues, fetal tissues or embryos, are highly expandable, and can be directed to differentiate into specific cell types. Furthermore, recent breakthroughs in cell reprogramming make it possible to take tissue biopsies from patients and reprogram the cells into pluripotent stem cells or specific cell types such as neurons or cardiomyocytes. This progress has allowed tissue engineers to have access to unlimited, immune-acceptable cell sources. To fully harness the therapeutic potential of stem cells, we need to understand how stem cells respond to microenvironmental factors including both biochemical and biophysical cues. This is not only required for controlling cell fate in vitro, but it is also important for the design of scaffolds and tissue constructs that can maximize the recruitment
of adult stem cells following implantation. In addition, to cultivate cells for clinical applications, quality control and FDA requirements must be fulfilled.

Tissue engineering using stem cells is an emerging and fast-growing field. There is a pressing need for a book that provides a comprehensive introduction to the field and summarizes its recent progress. We have invited experts in their respective fields to provide insightful reviews of specific topics on stem cells and tissue engineering. We hope that this book is timely and useful for researchers and students. Chapters 1 to 4 of this book introduce tissue engineering (Chapter 1) and discuss different types of stem cells (Chapters 2 to 4). Chapters 5 to 8 discuss the use of stem cells and biomaterials for the regeneration of cardiac tissue, blood vessels and the vascular network. Chapter 9 reviews the role of stem cells in general wound repair. Chapters 10 to 14 focus on skeletal tissue engineering, including cartilage, intervertebral disc and bone. Chapters 15 and 16 review the use of stem cells to treat spinal cord injury and neurodegenerative diseases. Chapters 17 and 18 illustrate state-of-art technologies used in stem cell engineering, including high-throughput systems and microtechnologies. Chapters 19 and 20 discuss quality control and regulatory issues. Although this book does not have the capacity to cover the use of stem cells for all tissues and organs, we hope that the general concepts and approaches illustrated in this book are helpful for researchers who are interested in other tissues and organs that are not discussed here.

We thank all the contributors for their hard work and valuable contributions. We also thank Joy Quek and the other staff of World Scientific Inc. for their tremendous effort in editing and organizing this book.

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1. Introduction

With the advent of the 21st century, the use of tissue engineering-based therapies to treat a variety of diseases and/or injuries has moved from being a dream of what might be possible in the future to the realm of the achievable. Even so, there is still much that needs to be done, much still to be learned; however, it is clear that major advances have been made in the last two decades.

The term tissue engineering is used here to describe a wide variety of approaches. This includes the replacement, the repair, and/or the regeneration of tissues and organs. Different terms have been used to describe this harnessing of the intrinsic biological abilities of the human body and of living cells. Although research in this general area goes back nearly half a century and the possibilities of such approaches was described even earlier, it was in 1987 that the term tissue engineering was introduced. Then in the 1990s the term regenerative medicine came into use. For some these terms are interchangeable. For others tissue engineering is used for
approaches that are aimed at fabricating substitute tissues outside of the body that then can be implanted into the body as replacements. For some the term regenerative medicine means stem cell technology. For this author, however, tissue engineering has a much broader meaning. Thus, to minimize any confusion associated with the choice of terms, it is the term tissue engineering that will be used in this introductory chapter and it will be used in the broadest sense to include replacement, repair, and regeneration, i.e. the wide variety of approaches that harness the intrinsic biological ability of the body and the use of living cells, whether of exogenous or endogenous origin.

It should be noted that over the past two decades the industry associated with this field has had its “ups and downs.” There were products being developed in the 1990s, and these were largely skin substitutes. The leading companies were Advanced Tissue Sciences (ATS) and Organogenesis (OI). As we entered the 21st century, however, we encountered what might be called “the sobering years.” Both ATS and OI entered bankruptcy. Today ATS no longer exists, but OI has reinvented itself and is a profitable company. In fact, in the last five years there has been a resurgence of the industry. In 2007, the last year for which there is data available, total industrial activity was US$2.4 billion with more than half of this being the sale of commercial products. For development stage funding the largest component is that of stem cells.

Whatever the approach being used in tissue engineering, a critical issue is the source of the cells to be employed. This thus will be addressed in the next section. One possibility of course is to use stem cells, and since the early reports of human stem cells a decade ago there has been a surge of activity. As stem cells and tissue engineering are the focus of this book, a brief introduction to stem cells is provided. To employ stem cells in a cell-based therapy will require, however, the translation of the basic benchtop science to the variety of applications that are possible. This is an area that has been largely overlooked, certainly not addressed to the extent necessary, and in the next to last section of this introductory chapter the issues that need to be addressed as one moves from the basic stem cell biology research to applications will be briefly discussed. The chapter then ends with some concluding comments.
2. Cell Source

Whatever the tissue engineering approach, whether it be one of replacement or that of repair and regeneration, a critical issue is that of cell source, i.e. from where will the cells come that are to be employed in the treatment or therapy. In addressing this issue, there are several questions that need to be asked. These are as follows.

- Will the source of cells be endogenous or exogenous?
- Will one use undifferentiated stem cells, progenitor cells, or fully differentiated somatic cells?
- Will one employ an autologous cell strategy or an allogeneic or even xenogeneic strategy?
- Are there differences associated with the age of the donor or with the disease state?
- Are there sex differences that must be taken into account?

Let us consider these one by one.

To start with, is the strategy one of recruiting cells from within the patient or one using an exogenous source? If the former, then the approach is an autologous one, and the challenge is how to recruit the cells. If the latter, then one must proceed to a series of additional questions.

This book focuses on stem cells; however, it also includes the use of progenitor cells that in fact can be derived from stem cells. Furthermore, and as will be discussed in the next section, there are different types of stem cells, e.g. embryonic versus adult, and these may be different in their ability to be differentiated into the particular type of cell to be used in the therapy. Even starting with a stem cell, however, does one use the stem cell directly in the therapy, does one use a progenitor cell derived from the stem cell, or does one use a fully differentiated cell?

One question for any clinical therapeutic strategy is that of autologous vs. allogeneic vs. xenogeneic cells. Although the use of autologous cells is attractive from the viewpoint of immunogenicity, the use of autologous cells does not in general provide for off-the-shelf availability to the clinician. Why is off-the-shelf availability important? For surgeries that must be carried out on short notice, e.g. following a heart
attack, off-the-shelf availability of the cells to be employed in the therapy is essential; however, even when the time of surgery is elective, one can argue that only with off-the-shelf availability will the wider patient population that is in need be served. There is of course one exception to the above generalization, and this is if the cells to be employed are to be recruited from within the body of the patient. In this case what is needed by the clinician off-the-shelf is in fact not the cells themselves, but perhaps only an acellular implant to be used in recruiting the cells and/or to serve as a target for the cells. In contrast, allogeneic cells or even xenogeneic cells do provide for off-the-shelf availability. Here the challenge is that of immunogenicity. The problem of achieving immune acceptance with xenogeneic cells is particularly severe; however, even for allogeneic cells for at least some cell types, e.g. vascular endothelial cells, a strategy for creating immune acceptance would have to be used.

Finally, there are the questions of differences due to age, due to the disease state of the patient, or due to the sex of the donor. Although largely unexplored, these can be significant issues, and it is important that future research addresses these questions. For example, there is a report that patient characteristics affects the number of human cardiac progenitor cells that will be available. There also is evidence that the disease state can have influence. In this case an example is that in patients with coronary artery disease there was observed a functional impairment of the hematopoietic progenitor cells.

There also are sex differences in the basic characteristics of cells whether they be stem cells, progenitor cells, or fully differentiated cells. This is a very important area, one which gives rise to a variety of questions. For example, if the cells are to be cultured, are different culturing protocols required for female cells as compared to male cells? For the clinical therapy itself, will the outcome be different depending on sex of the donor versus the sex of the patient? In this latter case, there are reports in the literature documenting differences.

There thus are a variety of questions to be answered, and although there is developing a rich literature, further research is required. In the next section, however, we move on to a very brief discussion of stem cells.
3. Stem Cells

As noted earlier, since the early reports in the late 1990s there has been a surge of activity and this increases at an ever accelerating level. Further details will be found in the literature\textsuperscript{12,13} and in subsequent chapters. Thus this section of this introductory chapter will only attempt to provide a broad and very brief overview of the different types of stem cells and other pluripotent cells available for use in tissue engineering. From an overall point of view, however, one may consider three general types of stem cells as follows.

3.1 Embryonic stem cells (ESCs)

These can be isolated from the inner cell mass of pre-implantation embryos during the blastocyst stage.\textsuperscript{3,4} They have the ability to differentiate into virtually all specialized cell types and thus are considered pluripotent. They also have the ability to proliferate in an undifferentiated state, i.e. they have the ability to self renew. Since different human ESC lines have been derived from different embryos, it is not surprising that different lines will exhibit different gene expression characteristics.\textsuperscript{14} Within this general category of stem cells there are in addition to those derived from embryos, those called embryonic germ cells (ECGs). These are derived from the gonadal ridge of a fetus that is five to ten weeks old,\textsuperscript{5–15} and these are primordial germ cells that \textit{in vivo} give rise to eggs or sperm in the adult.

3.2 Induced pluripotent stem (iPS) cells

These iPS cells are the result of the transformation of an adult, somatic cell through reprogramming into a pluriopotent stem cell.\textsuperscript{16,17} Although pluriopotent, these cells are not necessarily identical to ES cells even though they are similar. The reprogramming initially has been through retroviral transfection although there are a number of efforts in progress to carry out the reprogramming without the use of transfection. Like ESCs, iPS cells can lead to teratoma formation.
3.3 Adult stem cells

Adult stem cell populations have been found in many tissues of the human body. They are believed to be important to the repair mechanism intrinsic to many tissues and organs. They also in general are tissue specific; however, there are some exceptions. One of the exceptions to the believed tissue specificity of adult stem cells is the mesenchymal stem cell.\textsuperscript{6,7,18,19} This type of adult stem cell is derived from bone marrow stroma. In fact, one could view bone marrow transplantation as the earliest cell-based therapy. In vitro the MSC can differentiate into a variety of cell types. It is thus viewed as multipotent, but not pluriopotent or totipotent. Another exception to the general specificity of adult stem cells are the amniotic-fluid and placental derived stem cells.\textsuperscript{20} They have been shown to have the capacity for self renewal like ESCs, can give rise to numerous cell types, and have been characterized as having properties somewhere in between those of ESCs and adult stem cells. Finally, adult stem cells may be found in adipose tissue\textsuperscript{21} and also in the umbilical cord.\textsuperscript{22,23}

There clearly is much more that needs to be done to understand the characteristics of these different stem cell types and the factors involved in determining the differentiation pathway down which a stem cell can be directed. Just as the functional characteristics of a fully differentiated cell is orchestrated by a symphony of signals, the same can be said for the fate of a stem cell. This symphony includes soluble molecules, cell-cell contact, and the substrate/extracellular matrix to which the cell is adherent. It also includes what is of particular interest to this author and that is the role of physical or mechanical forces in modulating stem cell behavior. This includes the role of the physical force environment in the regulation or modulation of stem cell fate. As an example, we have shown that mouse ESCs in an early state of differentiation and when exposed to laminar flow and the associated shear stress exhibit an upregulation of endothelial cell phenotypic markers.\textsuperscript{24} This suggests that such physical forces, acting as part of a stem cell’s microenvironment, can participate in the direction of the differentiation process, perhaps even accelerate it. Taking a different approach, it has been demonstrated that mechanical stain inhibits the differentiation of human ESCs.\textsuperscript{25} Geometry is a different physical characteristic that can influence stem cell
differentiation. This has been demonstrated for MSCs.\textsuperscript{26} Interestingly, there also are reports that the mechanical properties of the extracellular matrix can influence stem cell fate.\textsuperscript{27} There also is the role of epigenetics in the regulation of stem cell fate. An example of this epigenetic role is the regulation of gene expression through histone modification or DNA methylation.\textsuperscript{28–30} Thus, there are a variety of ways in which the microenvironment can orchestrate stem cell fate, and we know very little about how to design the symphony of signals so as to optimize the outcome of this orchestration.

4. From Benchtop Science to Cell-Based Applications

If in fact stem cells are to be employed in a particular therapy or treatment, then one must address the translation of the benchtop research through a process that will result in the number of cells required for a specific application and one that will achieve regulatory approval.\textsuperscript{31,32} There are a number of aspects that must be considered, and in December 2008 Georgia Tech hosted a workshop on Stem Cell Biomanufacturing, bringing together a group of industry and academic researchers as participants. Some of the issues identified are as follows.

- The sourcing and isolation of the stem cell.
- The monitoring of stem cell phenotype.
- Possible inhomogeneity in the starting population.
- The expansion and propagation of the cells.
- The control of stem cell fate.
- Methods to assess genetic and epigenetic stability.
- Meaningful real time in-process assays.

All of this would need to be done with the quality control required for regulatory approval. This leads one to the concept of an automated cell processing facility. To develop such a facility would require research that leads to a knowledge base for process design. This would include having the ability to measure process variables in real time and experiments designed to determine functional relationships between process variables and what might be called product quality. There would
need to be robust strategies for the interrogation and evaluation of the variables affecting the processing of cells. There also would need to be an implementation of closed loop control methods. All of this would need to be supported by the use of multivariable statistical analysis to determine the variables affecting product quality. In addition, mathematical models relating the product and the process variables would need to be developed.

This of course means a different type of research on stem cells than what today is largely appearing in the literature. If we are to translate all the exciting advances taking place in our understanding of stem cells to applications including patient therapies, however, this is what will be required.

5. Concluding Comments

Tissue engineering, including replacement, repair, and regeneration, offers the hope that in the future we will be able to develop new therapies and treatments. This will be particularly important for diseases and injuries where currently there are no adequate treatments available. A critical issue is that of cell source, and here the variety of stem cells available have the potential of providing the answer. For each type of stem cell, however, there are issues that need to be addressed. Also, beyond the basic science there also will need to be research aimed at understanding how to optimize the processing of stem cells. Only with the combination of research on basic stem cell biology and research on stem cell processing will it be possible to translate the basic benchtop science into future applications and patient therapies.

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References


1. Introduction

Embryonic stem (ES) cells are pluripotent cells derived from the inner mass of mammalian blastocysts. They have the capacity to self-renew, while maintaining pluripotency: the ability to generate all cell types of the three germinal layers. Therefore, a variety of clinical applications have been proposed for ES cells, as well as in vitro studies of basic disease mechanisms, screens for drug discovery, and tissue engineering for degenerative diseases. However, ES cells are generic cells that are unrelated to the patient requiring treatment and the usage of human embryonic stem cells continues to be a contentious ethical issue.

Embryonic development and cellular differentiation are presumed to be unidirectional processes and cells undergo a progressive loss of pluripotency during cell fate specification. In the 1950s, classical experiments demonstrated that differentiated cells retain the genetic information required to revert to pluripotency\(^1\) and attempts were made to generate pluripotent stem cells functionally equivalent to ES cells from
differentiated cells (reprogramming) — including by somatic cell nuclear transfer, fusion of differentiated cells with pluripotent cells, application of pluripotent stem cell extracts to somatic cells, and direct in vitro adaptation of germ cells. Ultimately in 2006, Takahashi and Yamanaka showed that enforced expression of four transcription factors, Oct4, Sox2, Klf4 and c-Myc reprogrammed murine somatic cells to pluripotency. After this discovery, reprogramming drew enormous attention not just from stem cell biologists, but also from clinicians, bioengineers, geneticists, and developmental biologists. In this chapter, we review recent advances in the reprogramming field and discuss the potential future applications of induced pluripotent stem (iPS) cells.

2. Nuclear Reprogramming

Briggs and King showed that nuclei from Rana pipiens blastula cells have the ability to undergo normal cleavage and develop into complete embryos when transplanted into enclosed oocytes. This was the first demonstration that the oocyte cytoplasm contains factors that can reprogram the nuclei of differentiated cells back to a pluripotent state that allow donor cells to normally divide and develop into complete embryos. This finding was extended by a study by Gurdon and colleagues, who reported that even fully differentiated intestinal cells from Xenopus could be reprogrammed by frog oocytes. Although the efficiency was very low, these oocytes could give rise to adult animals indicating that the pluripotent state could be reacquired. Somatic cell nuclear transfer (SCNT) was not confirmed in other species until 1997, when Dolly the sheep was cloned. The discovery of reprogramming by SCNT led to further advances in the understanding of the epigenetic processes governing self-renewal and differentiation in murine and human ES cells.

Several groups demonstrated that fusing a somatic cell with an ES cell could induce pluripotency. This reprogramming can be accomplished when embryonal carcinoma (EC) cells, embryonic germ (EG) cells, or teratocarcinoma cells are fused with somatic cells. At the tetraploidy stage of the ensuing cell, the molecular program of the original somatic cell can be erased and epigenetically reprogrammed to that of an ES cells. With SCNT methods, reprogramming to a pluripotent state occurs within a few days and
can be greatly enhanced by co-expression of pluripotency-associated genes such as Oct4, Sox2, c-Myc, Klf4, Nanog, and Sall4. Yet, each of these methods has clinical limitations. SCNT has the advantage of using intrinsic reprogramming machinery in oocytes, but it requires the donation of human oocytes that can be in limited supply and raise ethical concerns. In somatic cell fusion, there are no ethical problems and no limitations on the supply of cells, but the processes result in the formation of the undesired tetraploid that is genetically unstable, and the overexpression of transcription factors increases the frequency of tumorigenesis in the resulting progeny.

In recent years, nuclear and cytoplasmic extracts from undifferentiated cells have been tried to induce the pluripotency of differentiated somatic cells. Extracts from pluripotent EG, EC cells or ES cells that contain the regulatory components needed for reprogramming can induce the pluripotency of somatic cells. The reprogramming extracts reversibly permeabilize the somatic cell and then induce transcriptional reprogramming, leading to epigenetic modification of the somatic cells. Even interspecies reprogramming via cell extracts showed some success in de-differentiating somatic cells. The extracts of regenerated new limbs induced partial dedifferentiation of c2c12-derived mouse myotubes into myoblasts and the extracts of *Xenopus* eggs induced the expression of the pluripotency genes Oct4 and Sox2 in mammalian somatic cells. When fibroblast cells were reversibly permeabilized and transiently exposed to extracts from mouse EC cells, they lead to Oct4 biphasic activation. Incubation of extracts from mouse ES cells with reversibly permeabilized NIH3T3 cells induced partial reprogramming. This approach to inducing pluripotency using pluripotent cell extracts could be an excellent alternative to nuclear transfer reprogramming due to the fact that eggs are not required and the resultant cells are diploid. However, the intrinsic difficulty to continue the exposure of the somatic cells to pluripotent cell extract holds yet the successful derivation of completely reprogrammed pluripotent stem cells.

Hence, a variety of studies have been proposed to better understand the reprogramming of pluripotent stem cells. However, the principal limitation to all the above technologies for cell reprogramming is that the generated pluripotent stem cells are unrelated to the patient who requires clinical treatment, and the use of human ES cells remains a contentious ethical issue.
3. Reprogramming by Defined Factors

Reprogramming somatic cells by using a defined set of factors is a novel concept and opens extraordinary possibilities for producing pluripotent stem cells without raising the ethical concerns associated with destroying human embryos.\(^5,6\) while allowing for patients to be treated with their own reprogrammed somatic cells. In their original report, Takahashi and Yamanaka retrovirally transduced different combinations of 24 candidate reprogramming genes into mouse embryonic fibroblasts derived from transgenic mice containing a neomycin resistance gene knocked into the Fbx15 locus.\(^3\) Cells that recovered neomycin resistance from the reactivation of the reporter gene were selected. They found that the retrovirus-mediated introduction of transcription factors was sufficient to reprogram mouse fibroblasts back to a pluripotent state. Specifically, the combined introduction of the four transcription factors, Oct4, Sox2, Klf4, and c-Myc were identified as sufficient to give rise to pluripotent cells, termed induced pluripotent stem (iPS) cells.

Selected iPS cells showed properties very similar to murine ES cells in morphology, proliferative characteristics, and expression of pluripotency markers. Moreover, iPS cells differentiated into all three germ layers \textit{in vitro}, and were able to form teratomas when injected into nude mice, confirming their pluripotency. Although Fbx15-selected iPS cells formed chimeras, they did not result in germ line transmission, raising the concern that iPS cells are not truly equivalent to ES cells. However, several studies have reported that iPS cells selected using Nanog or Oct4 are more epigenetically related to ES cells and can produce chimeras capable of germ line transmission.\(^11,12\) However, these iPS cells did not pass the most stringent pluripotency test: tetraploid complementation. Albeit negligible, the continuous expression of reprogramming genes seems to be responsible for the limited differentiation potential of the iPS cells, since the iPS cells produced by inducible lentiviral reprogramming factors succeeded to generate full-term mouse after tetracomplementation.\(^13,14\)

After the identification of murine iPS cells was reported, using the same four transcription factors, Oct4, Sox2, Klf4 and c-Myc or combined with novel factors, Nanog and Lin28, three different groups isolated human iPS cells.\(^15–17\) Human iPS cells from either combination of factors
shared defining characteristics with human ES cells, including gene expression profiles, morphology, proliferation, patterns of DNA methylation and histone modification, as well as telomerase activities. Human iPS cells were also able to differentiate into three germ layers in vitro as embryonic bodies and to form teratomas after injection into immune deficient mice.

4. Recent Advances in Reprogramming Methods

When the first iPS cells were isolated, many questions regarding the techniques to generate iPS cells were raised, such as how to identify iPS cells, how to derive iPS cells without potentially tumorigenic oncogenes, and how to generate iPS cells without using retro/lentivirus. Over the last three years, there have been many advances that have begun to overcome these hurdles.

Initially, Takahashi and Yamanaka created the first iPS cells using the neomycin resistant gene regulated by the pluripotent cell specific Fbx15 locus and claimed that reprogramming was a very inefficient process. Selection tools seemed essential to identify the iPS cells, and Nanog or Oct4 promoter-based drug resistant genes were used to help isolate murine iPS cells. Lately, murine and human iPS cells were derived by using the morphologies unique to ES cells. Furthermore, silencing of the retro- and lentivirus could be retrospectively used to identify the reprogrammed cells from the partially reprogrammed or transformed cells. The induction of the gene that mediates retroviral silencing in ES cells, Trim28, seems to be responsible for retroviral silencing during reprogramming in iPS cells.

The usage of oncogenic c-Myc and Klf4 in generating iPS cells is a major hurdle for potential clinical applications. One of the four reprogramming factors, c-Myc, is a well-established oncogene, and its continued expression and/or potential reactivation in iPS cell derivatives is not suitable for future iPS cell therapies. Indeed, head and neck tumor formation has been observed in iPS cell-derived chimeric mice possibly due to the reactivation of c-Myc. However, c-Myc is dispensable for reprogramming murine and human somatic cells, although its absence significantly reduces the reprogramming efficiency. Some somatic cells highly express
endogenous reprogramming genes and may not need the expression of all reprogramming factors. Indeed, neural progenitor cells (NPCs) that highly express endogenous Sox2, and can be reprogrammed without the ectopic expression of Sox2.\\textsuperscript{22} Furthermore, even introduction of Oct4 alone was shown to be sufficient to generate iPS cells from NPGs.\\textsuperscript{23,24} Similarly, meningiocytes and keratinocytes appear to be particularly prone to reprogramming due to their relatively high endogenous expression of Sox2, Myc and Klf4.\\textsuperscript{25,26} These results indicate that the reprogramming cells like NPCs would require a minimal genetic modification in reprogramming and result in most desirable safe iPS cells.

Limitation to the current methods in generating patient specific iPS cells is the residual presence of the reprogramming factors in chromosome. Retro/lentiviral integration into the genome carries a risk of tumor formation when random integration activates pathways for cell proliferation or inhibits the tumor suppressor pathways. Using methods based on non-integrating vectors or direct exposure to reprogramming proteins is desirable. Stadtfeld and colleagues were able to generate iPS cells from adult mouse fibroblast and liver cells using non-integrating adenoviral vectors.\\textsuperscript{27} They demonstrated that adenoviral-mediated transient expression of the exogenous reprogramming factors eliminated the risk of insertional mutagenesis. Okita and colleagues also successfully generated iPS cells without using any viral vectors but multiple transient transfection of the reprogramming factors.\\textsuperscript{28} Lately, oriP/EBNA1-based episomal vector was successfully used to generate human iPS cells.\\textsuperscript{29} All the non-integrating vector methods provide a safer iPS cells but suffer from the extremely low reprogramming efficiency.

Usage of three or four transcription factors for reprogramming results in the high incidence of multiple integration of reprogramming factors into chromosome. Combining the reprogramming factors with picornaviral 2A sequences allowed the expression of multiple genes in one backbone.\\textsuperscript{30,31} This method generated iPS cells through less number of retro- or lentiviral insertion into the genome, as opposed to using separate viral vectors for each reprogramming factor. Because the single viral copy may also be removed from the iPS cell genome after reprogramming (e.g. by loxP/Cre technology) the authors successfully generated safer iPS cells.\\textsuperscript{32} Similarly, Kaji and colleagues developed a piggyBac transposon-based
vector expressing four genes enabling the creation of transgene-free iPS cells following removal of the transposon.\textsuperscript{33} Despite these advancements, the concern lingers that all of these factors have links to tumorigenesis.

An alternative approach that allows the complete avoidance of the use of oncogenes altogether is to use small molecules instead. Shi \textit{et al.} generated iPS cells from NPCs by transduction of Oct4 and Klf4, and found that simultaneous treatment with G9a inhibitor, BIX-01294, remarkably increased the reprogramming efficiency.\textsuperscript{34} Subsequent studies have confirmed that epigenetic modification or the activation of self-renewing signaling through small molecules can improve reprogramming. Additionally, the histone deacetylase (HDAC) inhibitor valproic acid (VPA) and Trichostatin A (TSA), as well as the DNA methyltransferase inhibitor, 5-aza-cytidine have been shown to increase reprogramming efficiency.\textsuperscript{35,36} The Wnt signaling component WNT3a, or the L-channel calcium channel agonist Bayk8644 (BayK) also can increase the reprogramming efficiency.\textsuperscript{22} In another study, the inhibition of mitogen-activated protein (MAP) kinase signaling and synthase kinase-3 (GSK3), allowed reprogramming to occur, even in the absence of Sox2 and c-Myc.\textsuperscript{37} Recently, tumor suppressor pathways, including p53, Ink4a/Arf and p21, were shown to play a role as a barrier to reprogramming.\textsuperscript{38–41} These several signaling pathways are well known to facilitate the ES cell self-renewal or cell proliferation and seem to increase the reprogramming efficiency.

Ultimately, iPS cells have been generated without using vectors at all, but rather by directly introducing proteins into fibroblasts. Zhou and colleagues purified recombinant reprogramming factors fused with the poly-arginine (i.e.11R) protein transduction domain of the C-termini and were able to generate iPS cells from mouse embryonic fibroblasts.\textsuperscript{42} Kim and colleagues established 293T cell lines stably expressing reprogramming factors fused with the poly-arginine tag and demonstrated the formation of human iPS cells by exposure of the cell extract to fibroblasts.\textsuperscript{43}

\section{5. Future Perspectives on Reprogramming and iPS Cells}

Dr. Yamanaka’s reports of generating iPS cells using defined factors revolutionized the approach to manipulating the cellular identity. Initiated by the finding that fibroblasts became induced to the myogenic lineage by
expressing a given myogenic factor, dedifferentiation or reprogramming to different cellular fates have been explored, including hematopoietic, pancreatic and cardiac lineages. Pluripotent iPS cells generated via reprogramming will have a broader applicability, since they can differentiate into any cellular lineage. They can be used to investigate the disease progression in vitro, and to provide a platform to screening chemicals for genetic disorders (Fig. 1). Recent advancement of high throughput sequencing, when combined with iPS cells, will allow us to investigate the early development of genetically defined, personalized cells in vitro.

After human ES cells were isolated, they have been used to make models of human diseases. Human ES cells genetically modified by either overexpression or knock-down of genes of interest was proposed to mimic the early embryonic cells of human diseases in vitro. In vitro differentiation of ES cells were presumed to follow the early developmental program of normal embryonic development. iPS cells derived directly

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**Fig. 1.** Recent advances and future perspectives of reprogramming. Since the Yamanaka lab reported the generation of iPS cells in murine fibroblasts, there has been an explosion of research on factor-based reprogramming. 1) iPS cells were generated without using integrating retro- and lentiviral vectors. Small molecules were identified that increased reprogramming efficiency. 2) Reprogrammed iPS cells will be used to generate in vitro disease model. 3) In vivo transdifferentiation will be a practical alternative to pluripotent stem cells for genetic or non-genetic degenerative diseases. Shown in red are challenges that will lead to successful utility of the iPS cells in regenerative medicine.
from patients provide an in vitro model that will more closely mimic the pathology of the disease. Following are examples of the rigorous attempts to generate human disease models using murine and human iPS cells.

Murine iPS cells have been used to illustrate the therapeutic potential of iPS cells in vivo. For example, Hanna and colleagues showed that transgenic mice engineered to have human sickle cell anemia could be successfully treated with hematopoietic progenitor cells produced from autologous iPS cells and genetically repaired. Wernig et al. showed that iPS cell-derived neuronal cells could integrate into fetal brains and ameliorate the symptoms of rats with Parkinson’s disease. Since then, more studies have reported the derivation of therapeutically relevant cell types from iPS cells, including human insulin-secreting cells, and functional mouse and human cardiomyocytes, as well as mouse endothelial cells that successfully treated mice with coagulation disorder hemophilia A.

Human iPS cells from patients’ fibroblasts were generated to investigate human diseases in vitro. Our group has generated iPS cells derived from a variety of genetic disorders, including adenosine deaminase deficiency-related severe combined immunodeficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS), Gaucher disease (GD) type III, Duchenne (DMD) and Becker muscular dystrophy (BMD), Parkinson disease (PD), Huntington disease (HD), juvenile-onset, type 1 diabetes mellitus (JDM), Down syndrome (DS)/trisomy 21 and the carrier state of Lesch-Nyhan syndrome. iPS cells generated from patients of neuromuscular diseases, including amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA), were shown to differentiate into motor neurons, providing a novel in vitro motoneuron disease model. A range of iPS cells from Parkinson’s patients was also generated as an invaluable resource for studying the disease. These cells do not contain transgenic reprogramming factors in their genomes as the factors have been excised by Cre recombinase from the integrated lentiviral vector. Recently, Lee and colleagues extended the iPS cell technology to model autosomal recessive familial dysautonomia (FD) in vitro, and demonstrated the FD-related mis-splicing of IKBKAP and concurrent defects in neurogenic differentiation and migration behavior. Most of iPS cells used to model human diseases still contained the retroviral vectors in their chromosomes. With the advent of reprogramming
methods not relying on integrating virus, iPS cells are expected to be more close to ES cells and make better disease models.\textsuperscript{43}

Although it is highly hoped to utilize iPS cells in various applications mentioned above, there are several issues to be resolved (Fig. 1). First of all, methods to generate clinically useful iPS cells should be improved. Non-integrating virus, direct protein transduction, and nuclear transfer will make genetically non-modified iPS cells. But, their reprogramming efficiency is extremely low, and will not be practical to produce iPS cells in a reliable manner. Improvement in viral vectors, or screening small molecules will be essential to optimize the reprogramming methods.\textsuperscript{62,63}

In order to reach the final goal of regenerative medicine using pluripotent stem cells, it is crucial to make them acquire the desired lineage \textit{in vitro}. Directed differentiation of pluripotent stem cells relied on the previous knowledge of cell specification obtained from developmental biology.\textsuperscript{51} Treatment of cytokines, growth factors, chemicals, and small molecules have been attempted to differentiate pluripotent stem cells. Ectopic expression of lineage specific transcription factors is a reliable approach to direct the differentiating cells into specific lineage.\textsuperscript{64,65} Cell surface markers were used to isolate the cells of interests. When mouse ES cell lines expressing fluorescent proteins of lineage specific markers are available, they provide an effective way to isolate pure cell population. The isolation of lineage specific cells from human ES cells was facilitated by the transgenic human ES cell lines with lineage markers. Teratoma formed by the incompletely differentiated pluripotent stem cells continuously impedes the utility of cells differentiated from ES cells. Improvement of negative selection of partially or non-differentiated cells, or rigorous isolation of completely differentiated cells are required. \textit{In vivo} transdifferentiation will be an alternative to iPS cells of great practical importance. Most of the degenerative disorders show loss in parenchymal or functional cells in tissues: loss of endocrine beta cells in type I diabetes, dopaminergic neurons in Parkinson’s diseases and motoneuron in ALS. Despite the degeneration of these cell types, the stromal or adjacent cells surrounding cells are still intact. When expressed with a combination of genes important for the development of the same lineages of the degenerated cells, the surviving cells in the damaged tissue will change their cellular fate to the cells of the interest and will help
to recover the function. Given the great opportunity of manipulating cell fates, it becomes possible to conquer the devastating diseases, but there are still much to overcome.

Acknowledgments

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1. Introduction

The mammalian hematopoietic system is a dynamic organ, containing mature cells that function in the processes of adaptive and innate immunity, blood clotting, and oxygen transport. These mature cells have a limited lifespan and are replenished by various blood lineage-specific progenitor cells, which in turn are replenished by hematopoietic stem cells (HSCs). Thus, it is the HSC population that serves as the foundation of the system, giving rise to all mature hematopoietic lineages throughout the lifetime of an organism. To perform this specialized function, HSCs have two concomitant properties that make them unique; the ability to differentiate to give rise to all mature hematopoietic cell types and the unlimited ability to divide to produce daughter HSCs that maintain the stem cell pool, a process termed self-renewal.

HSCs were the first stem cells to be identified, by Till, McCullough and Siminovitch, in 1963. Over the last 46 years, a great number of elegant studies in the field of HSC biology have characterized the timeline of emergence and locations of these cells during mammalian development, the combination(s) of cell surface antigens that can be used to identify
and/or prospectively isolate HSCs, and have developed advanced \textit{in vitro} and \textit{in vivo} assay systems to reliably evaluate the function of these cells. As our understanding of the biology of HSCs has progressed, so have clinical therapies utilizing these specialized cells. The first successful bone marrow (BM) transplants as treatment strategies for hereditary immunodeficiency and acute leukemias were performed in the late 1960s and early 1970s (reviewed in Ref. 3). Now, forty years later, transplantation of HSCs is being developed and/or successfully applied toward hematological diseases, hereditary metabolism disorders, congenital immunodeficiency, diseases of the central nervous system, solid tumors and lower extremity ischemic disease.\textsuperscript{4}

Solutions to the immediate and future challenges faced in clinical HSC transplantation will depend on developments in the field of tissue engineering, including methods to expand available sources of HSCs and to differentiate pluripotent cell lines into HSCs for clinical utility. In this chapter, we will discuss available sources of HSCs (adult, fetal and embryonic), current and future clinical applications of HSCs, and the challenges that can be addressed by tissue engineering.

2. Hematopoietic Stem Cell Sources

2.1 Adult

The first HSC source used in clinical therapy was adult BM, the site in which HSCs were first identified and isolated from.\textsuperscript{1} As clinical BM transplantation has been a reality for approximately 40 years, a large amount of clinical data has been amassed that clearly demonstrates the successes and limitations of this therapeutic strategy (Table 1). More recently, it was discovered that HSCs can be mobilized from the BM into the peripheral blood by stimulation with hematopoietic growth factors\textsuperscript{5} or myelosuppressive therapy.\textsuperscript{6} Mobilized peripheral blood (M-PB) has been used successfully in lieu of BM to reconstitute hematopoietic and immune function in patients, with the major advantages that it is a less invasive harvesting method and typically results in shorter engraftment time, thus reducing complications.

One of the major limitations in allogeneic BM or M-PB transplantation, in which the donors are non-identical to the patients, is the availability of
histocompatible donors. Typically, suitable donors are family members fully matched with the patient in expression of human leukocyte-associated antigens (HLA) or differ in one HLA antigen only. Establishing large, international donor volunteer registries has made progress toward a solution for the lack of compatible donors. A second limitation/risk to consider in use of BM or M-PB for transplantation is the common complication of graft-versus-host disease (GVHD), where the immune cells from the donor BM or M-PB recognize the patient’s cells as foreign and mount an immunologic attack. Treatment of GVHD involves immune suppression, potentially raising the risk of infection and, in cancer therapy, risk of relapse of disease.

2.2 Fetal

Umbilical cord blood (UCB), blood that remains in the umbilical cord after childbirth, has been widely accepted as a rich source of HSCs. Since

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<tr>
<td><strong>Fetal</strong></td>
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<tr>
<td>Umbilical cord blood</td>
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<tr>
<td><strong>Embryonic</strong></td>
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<td>Embryonic stem cells</td>
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<td>iPS cells</td>
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the first human UCB transplant performed 20 years ago, more than 10,000 UCB transplants have been successful in both pediatric and adult patients (reviewed in Ref. 9). The placenta itself has more recently been shown in mouse and human to be a potent source of HSCs, however, this source has yet to be clinically utilized. Importantly, cord blood banks have been established worldwide for the collection and cryopreservation of UCB for allogeneic transplantation. This bank represents an important distinction and advantage over the donor BM registry, that is, UCB can be frozen and banked for future thaw and immediate use while adult BM must be freshly harvested for transplant (Table 1).

UCB, and potentially the placenta, represent valuable alternative sources for patients who require HSC transplantation but have no readily available HLA matched donor, as this type of transplant permits a greater degree of HLA mismatching without an unacceptably high incidence of GVHD. Patients and UCB units can differ in two HLA antigens, however, the required cell dose must be more than $2.5 \times 10^7$ cells per kilogram of the patient. This caveat, the available cell dose per unit of UCB, greatly limits the broader use of UCB transplantation in adult patients. Recent developments to overcome this cell dose issue include transplanting two UCB units per patient, intrabone injection of UCB cells, and ex vivo expansion of HSCs, which will be discussed further below.

2.3 Embryonic

Human embryonic stem (ES) cells, first derived by James Thomson et al. in 1998, have received a large amount of attention owing to their great potential in regenerative medicine. These cells are derived from the inner cell mass of early human blastocysts, can be expanded and propagated indefinitely in culture, and have the potential to differentiate to generate cells of any tissue type. Ethical controversy surrounding the use of human blastocysts to derive ES cell lines has been bypassed by the discovery that it is possible to derive induced pluripotent stem cells (iPS cells) from adult human fibroblasts. Methods to reprogram adult cells into embryonic-like cells have been reliably reproduced by many groups and offer the potential of patient-specific cellular therapies. While the therapeutic
potential of iPS cells is great, it remains unclear as to the precise similarity of these cells to human ES cells. As such, continuation of studies utilizing both of these cell types, preferably in parallel, is ideal. A recent change in the US federal policy to permit federal funding for a greater number of human ES cell lines will certainly accelerate momentum in this field.20

The ability to differentiate human ES and iPS cells into HSCs for clinical use would have unique advantages, including the ability to engineer these cells to be drug resistant, allowing more specific and effective administration of chemotherapy in cancer treatment, and would permit establishment of hematopoietic chimerism to promote immune acceptance of other tissues derived from the same source of pluripotent cells.20 While in principle, using human ES or iPS cells as a cell replacement source for HSCs and other lineages is a thrilling prospect, it is deceptively simple. The feasibility of using these cells in a clinical setting is unclear and we do not yet understand how to differentiate these cells in a controlled and precise manner21 (Table 1). Clearly, human ES and iPS cells are able to spontaneously develop into cell types representing all three germ layers in vivo, as shown by teratoma formation, but the cellular and molecular mechanisms controlling these processes are incompletely understood.21 Thus, attempts to direct differentiation of human ES cells into HSCs in vitro have amounted to very limited success and heterogeneous results over the past 25 years.22 Certain human ES cell-derived hematopoietic cells are observed to have HSC-like properties, however, these cells do not represent functional HSCs capable of long-term, clonal, multilineage hematopoietic reconstitution of mice or larger animal models.23–26

While some groups have developed protocols to differentiate human ES cells into more mature hematopoietic lineages, including erythroid,27–30 myeloid31,32 and lymphoid cells,33–35 few have demonstrated that these protocols actually direct cells to the hematopoietic fate as opposed to creating conditions permissive to spontaneous differentiation or expansion of spontaneously differentiated hematopoietic cells.21 Development of methods to reliably and efficiently create HSCs from human ES or iPS cells will represent a significant leap towards their clinical use. This will depend on development of culture techniques to more closely mimic the in vivo
microenvironment needed to stimulate hematopoietic specification of pluripotent stem cells, as discussed further below.

3. Applications

Clinical HSC transplantation has classically been utilized for replacement of hematopoietic cells that have otherwise been damaged by a particular genetic condition, disease, or exposure to chemotherapy or radiation. A long history of clinical success in treatment of conditions such as hereditary immunodeficiency, leukemia/lymphoma, and aplastic anemia has been documented. Use of HSC transplantation to replace other cell types, such as in cerebral palsy, type I diabetes, neurological damage, endocrine disease, and spinal cord injury, has recently been proposed and debated (reviewed in Ref. 12). The possibility of using HSCs in non-hematopoietic cell replacement therapy was bolstered a number of years ago by several reports demonstrating remarkable regenerative potential of HSCs in animal models of heart infarct, stroke, spinal cord injury and liver damage. A contrasting study has definitively demonstrated that transplantation of a single HSC was able to robustly reconstitute the hematopoietic system of mice but did not contribute to non-hematopoietic tissues including the brain, kidney, gut, liver or muscle. This work suggests that differentiation of circulating HSCs and/or their progeny into non-hematopoietic organs is an extremely rare event, if it occurs at all.

In resolving these seemingly conflicting reports, it is an interesting and tempting hypothesis to consider that the role of HSCs or their progeny in repair of damaged organs is not necessarily due to direct differentiation, but rather the release of trophic factors that are able to stimulate repair. In fact, observed beneficial effects of UCB transplantation include reduced inflammation, protection of nervous tissue from apoptosis and nerve fiber reorganization. UCB transplantation for type I diabetes, cerebral palsy, brain injury and endocrine disease is in the process of being transitioned from the lab to the clinic, with numerous patients being treated in clinical trials (reviewed in Ref. 41). The clinical benefit of such therapies, and the mechanism behind them, remain to be discovered. Certainly, clinical HSC transplantation for hematopoietic replacement will continue to achieve
greater and greater success as we improve transplant protocols and methods to expand sources of HSCs.

4. Challenges for Tissue Engineering

There are a number of challenges faced in clinical HSC transplantation that have great potential to be addressed and solved by tissue engineering, including methods to expand currently available sources of HSCs and methods to differentiate pluripotent stem cells (human ES and iPS cells) into HSCs for clinical utility. One of the major issues that directly applies to both of these applications surrounds the ideal method for evaluating and enumerating HSCs. It has been well established in the field of HSC biology that these cells are most rigorously defined functionally, through demonstration of their ability to self-renew and differentiate into all blood lineages in vivo. The NOD/SCID mouse xenotransplant assay, whereby human HSCs are evaluated for their ability to reconstitute the blood system of non-obese diabetic/severe combined immunodeficient mice, has become widely accepted as a surrogate assay for human HSCs. More recently, it has become clear that mice with greater degrees of immunodeficiency are more sensitive hosts for human HSC engraftment. Thus, it is debated whether the xenotransplant assay is a true measure of human HSC function as opposed to a measure of immunologic escape from rejection. Primate studies suggest that the cells which contribute to NOD/SCID reconstitution represent short-term repopulating cells rather than true HSCs. Likely, novel methods developed to expand human HSCs as shown in mouse xenotransplant models will need to be confirmed in higher-order primates prior to their use in clinical therapy.

4.1 HSC expansion

HSCs require a complex microenvironment to retain their stem cell properties. In the BM, HSCs are surrounded by bone matrix and cells including osteoblasts, mesenchymal stem cells, fibroblasts and adipocytes, each of which produces various cytokines and growth factors. Ultimately, the goal of HSC expansion is to mimic this microenvironment in order to obtain sufficient numbers of HSCs to reconstitute long-term hematopoiesis from
adult, fetal, or embryonic sources (Fig. 1). Different culture medias, growth factors and supplements have been widely tested.\(^\text{43}\) Since the early 1980s, many hematopoietic cytokines were reported to be able to expand HSCs \textit{in vitro} without significant alteration of their primitive activity, however, it is now clear by xenotransplant assay that traditional culture of HSCs compromises their \textit{in vivo} potential.\(^\text{4}\) In addition, early attempts to move these preclinical results to patients failed to show improvements in engraftment time after HSC transplantation.\(^\text{44}\) Clearly, it is difficult to genuinely simulate the hematopoietic microenvironment \textit{in vitro} and culture of HSCs has historically introduced defects that promote apoptosis,\(^\text{45}\) disrupt homing of HSCs to the BM,\(^\text{46,47}\) and exhaust their self-renewal capacity.\(^\text{48}\)

In consideration of novel systems and methods for HSC expansion, \textit{ex vivo} HSC culture should be a closed bioprocess to avoid contamination and should attempt to achieve the greatest level of expansion of HSCs with the lowest level of manipulation. Traditional static cultures (including flasks and plates) may not be the most suitable, as they do not provide a
proper three-dimensional environment to achieve nutrient and oxygen distribution. Recently, a static bioprocess consisting of two gas permeable culture bags, separated by a magnetic system to eliminate undesired cells, was shown to promote expansion of human HSCs. A number of other new protocols for HSC expansion are in development, involving modified cytokine cocktails, co-culture methods and bioreactors (Fig. 1). Most \textit{ex vivo} culture conditions include the cytokines interleukin-3 (IL-3), IL-6, granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF), and Flt-3 ligand (FLT3L). In terms of novel cytokines to add to this cocktail, angiopoietin-like 5 (ANGPTL5) and insulin-like growth factor binding protein 2 (IGFBP2) have recently been shown to expand human UCB HSCs as assayed by NOD/SCID xenotransplant. Presently, several groups are testing tetraethylenepentamine (a copper chelator), histone deacetylase inhibitors and DNA methylation inhibitors in human HSC culture systems, with promising results. In addition to cytokines and pharmacological inhibitors, many studies have defined key signaling pathways that regulate normal development and expansion of HSCs \textit{in vivo}, including Notch, Wnt/β-catenin, Hedgehog, and BMP signaling (reviewed in Ref. 55). This knowledge will also be important to apply in the development of protocols to expand HSCs \textit{ex vivo}.

Co-culture of human HSCs with a supporting layer of cells provides an alternative strategy to liquid culture-based expansion, in an attempt to mimic cellular networks, direct cell-cell contacts, and soluble regulatory proteins found \textit{in vivo}. In vitro and \textit{in vivo} studies have shown that these support cells (also called stromal cells) can provide signals to control proliferation, survival, and differentiation of HSCs. Mesenchymal stem cells (MSCs) provide the most popular source of supporting cells. MSCs can be isolated from a variety of fetal and adult tissues, and have been used in preclinical models as a method to maintain HSCs in a primitive state.

Finally, bioreactors offer a modern, tissue engineering-based approach to model the hematopoietic microenvironment with a relatively stable oxygen concentration, pH and nutrient supply. Diverse bioreactors with specific characteristics have been designed for \textit{ex vivo} HSC expansion, including stirred and perfusion systems, rotating wall vessels, and those that minimize shear stress (reviewed in Ref. 43). With the growing range of clinical applications of HSCs and lack of efficient tools to expand them,
novel developments in *ex vivo* culture and tissue engineering offer great promise for the future of clinical HSC expansion.

### 4.2 Differentiation of pluripotent stem cells to HSCs

As highlighted above, the differentiation of human ES or iPS cells into HSCs remains a technical challenge. In mouse ES cells, this challenge has been met with some success through overexpression of the genes *Cdx4* and *HoxB4*,\(^{57,58}\) which permit derivation of a limited number of functional HSCs (Fig. 2). Unfortunately, however, this strategy does not directly...
apply to human ES cells. Current progress in differentiation of HSCs from human ES cells have relied on co-culture with supportive cells,\textsuperscript{24,25} formation of embryoid bodies,\textsuperscript{23} or these methods in combination. Clearly, the limited success of the current differentiation strategies indicates that key signals necessary for full developmental maturation of cultured ES or iPS cells into HSCs must be missing.

New experimental approaches toward hematopoietic differentiation from pluripotent stem cells are desperately needed. These may include use of some the methods described above for HSC expansion, however, there are several distinctions from HSC expansion studies that need to be taken into account. One important consideration is the potential biological difference between human ES and iPS cells, which may be reflected in their ability to differentiate into HSCs and/or the signals required to induce this. It has been suggested that iPS cells, as they are derived from adult tissues, have a greater cellular memory and as such may be more amenable to directed differentiation into HSCs or other specific cell types.\textsuperscript{59} A second, critical consideration is that residual undifferentiated ES or iPS cells pose a risk of teratoma formation in patients. Thus, differentiation protocols need to be highly efficient in formation of HSCs, or must include steps to purge residual undifferentiated ES or iPS cells from HSCs. In emphasis of this point, it has been found that unfractionated mouse ES cells, following differentiation by co-culture with OP9 stromal cells and injection into the fetal liver of monkeys, caused teratomas in the thoracic or abdominal cavities of all recipients.\textsuperscript{60}

The ability to generate fully functional human ES or iPS-derived HSCs capable of long-term multilineage reconstitution of animal models and ultimately patients remains a challenge and will depend upon developments in tissue engineering as well as further understanding of the intrinsic gene regulation and extrinsic environmental cues that control these developmental processes \textit{in vivo}.

\textbf{Acknowledgments}

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References


Mesenchymal Stem Cells for Tissue Regeneration

Ngan F. Huang and Song Li

1. Introduction

In the past two decades, significant progress has been made in the field of stem cell research. An important finding is that adult stem cells harbor greater regeneration potential and plasticity than what was previously thought. This discovery has led to tremendous interest in developing methods to direct stem cell differentiation into lineages for the therapeutic delivery into diseased or dysfunctional tissues. Among the adult stem cells, mesenchymal stem cells (MSCs) are a promising therapeutic cell source due to the ease of isolation, high proliferative capacity, and multipotency.1 MSCs can be found in numerous tissues of the adult mammal and can be harvested in a reproducible manner. Since large quantities of these cells can be cultured in vitro, it is feasible for MSCs to be delivered directly in vivo or incorporated into tissue engineered constructs before transplantation. These two approaches have been explored for treating a wide range of diseases or traumatic events, including myocardial infarction, peripheral arterial disease (PAD), spinal cord injury and skin wounds.
By developing robust methods of differentiating MSCs into therapeutic cells of interest and organizing the cells into functional three-dimensional tissues, it may be possible to fulfill the potential of MSCs for clinical use. This review aims to provide an overview of some therapeutic applications for MSCs in tissue engineering and regenerative medicine.

2. MSC Sources and Phenotype

MSCs can be generally defined as adherent and elongated cells that reside in mesenchymal tissues and can self-renew as well as produce progeny with more specialized function. MSCs have been observed and purified from numerous origins, including bone marrow, adipose tissue, skeletal muscle, blood, liver spleen and dental pulp. Among them, bone marrow and adipose tissue are perhaps most characterized origins of MSCs, and this review will focus on MSCs derived from these two sources.

2.1 Bone marrow MSCs

Although MSCs account for only 0.01% among total nucleated cells in the bone marrow, they have over million-fold expansion capability and multilineage differentiation potential. Bone marrow MSCs are easily harvested by aspiration from the iliac crest. Phenotypically, there is no unique marker that specifically identifies bone marrow MSCs. Consequently, MSCs are characterized based on the positive expression of numerous cell surface antigens such as CD29 (integrin β1), CD44 (receptor for hyaluronic acid and matrix proteins), CD105 (endoglin), and CD166 (cell adhesion molecule). On the other hand, they do not express markers typically associated with hematopoietic lineage, such as CD14 (monocyte surface antigen), CD34 (hematopoietic stem cell surface antigen) and CD45 (leukocyte surface antigen). Due to the differences in characterization methods, the International Society for Cellular Therapy recommended the designation of these cells as multipotent mesenchymal stromal cells and proposed the following minimal criteria for MSC designation: adherence to plastic dishes; phenotypic expression of CD105, CD73 and CD90; lack of surface molecule expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and class II major histocompatibility
complex antigen (HLA-DR); and differentiation capacity into osteoblasts, adipocytes and chondroblasts in vitro.\textsuperscript{11}

Based on the cell surface antigens, bone marrow MSCs can be isolated using fluorescence activated cell sorting (FACS) or magnetic activated cell sorting (MACS). Other methods to purify MSCs include Percoll gradient centrifugation and their selective adherence onto tissue-culture treated Petri dishes. To maintain their proliferative capacity, the purified bone marrow MSCs can be expanded in culture media containing defined serum-free components (i.e. StemPro\textsuperscript{®} MSC Serum Free Medium, Invitrogen, Carlsbad, CA) or pre-screened fetal bovine serum. Under these growth conditions, bone marrow MSCs can be cultured for more than eight passages.

\section*{2.2 Adipose-derived stem cells (ASCs)}

According to the nomenclature established by the International Fat Applied Technology Society, ASCs are adipose-derived MSCs that are adherent in plastic culture dishes.\textsuperscript{12} These cells were first reported by researchers who showed that cells dissociated from collagenase-treated human adipose tissue gave rise to multipotent cells that can differentiate into the cells of adipose tissue, cartilage and bone.\textsuperscript{13} These cells were later confirmed to give rise to other cell types, including endothelial cells (ECs), smooth muscle cells (SMCs) and cardiomyocytes.\textsuperscript{14–16} To isolate ASCs, adipose tissue derived from liposuction is digested with collagenase and then centrifuged to separate the stromal population in the lower layer of the pellet from the adipocytes in the upper layer.\textsuperscript{17} Like bone marrow MSCs, ASCs can be purified based on the expression profile of surface marker antigens using FACS or MACS. ASCs can be maintained in defined serum-free media (i.e. MesenPRO RS Media, Invitrogen, CA) as well as serum-containing media.

Although ASCs and bone marrow MSCs have greater than 90\% similarity in immunophenotype,\textsuperscript{18} there are some reported differences in surface antigen expression. For example, CD14 and HLA-DR was reported to be absent in bone marrow MSCs, but have been identified in early passage human ASCs at low frequency.\textsuperscript{19} Furthermore, ASCs appear to have temporal changes in immunophenotype with subsequent
passaging. However, these differences in immunophenotype could also be attributed to differences in species or the methods of isolation, purification, or detection.

3. Differentiation of MSCs *in vitro*

For tissue engineering and regenerative medicine applications, one strategy is to differentiate the cells *in vitro* into lineages of interest before delivering them *in vivo* for therapeutic treatment. Bone marrow MSCs and ASCs have been shown to differentiate into a variety of lineages, including myogenic, osteogenic, chondrogenic, and adipogenic lineages. A number of strategies to direct their differentiation have been used, including the use of soluble factors, mechanical stimulation, extracellular matrix (ECM) factors and genetic engineering approaches, which are briefly discussed below.

One of the most commonly used strategies to induce differentiation is by the addition of soluble factors, such as growth factors and small molecules. Using soluble factors, bone marrow MSCs and ASCs have a high propensity to differentiate into cells of mesenchymal lineage, including bone, adipose tissue and cartilage. Osteogenic differentiation can be induced by culturing the cells in the presence of dexamethasone, ascorbic acid, and \( \beta \)-glycerophosphate, whereas adipogenesis is enhanced by 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin. Chondrogenic growth factors include transforming growth factor-\( \beta \)3 (TGF-\( \beta \)3) and bone morphogenetic protein-6 (BMP-6). Besides osteogenic, adipogenic, and chondrogenic lineages, MSCs and ASCs have been shown to differentiate towards other lineages at lower yields. For example, platelet-derived growth factor (PDGF) and TGF-\( \beta \)3 stimulate smooth muscle phenotype, whereas 5-azacytidine treatment induced the formation of cardiac-like cells that expressed cardiac markers \( \beta \)-myosin heavy chain, desmin and \( \alpha \)-cardiac actin.

Besides soluble factors, mechanical stimulation is another potent regulator of cell behavior and function. Physiologically, mechanical stimulation plays an integral role in cell phenotype. For example, blood vessels experienced fluid shear stress and cyclic strain due to the pulsatile flow generated by the beating heart, and bone is subjected to compressive
loading from gravitational forces. Due to the importance of mechanical factors for physiological maintenance, the role of mechanical factors on MSCs and ASCs differentiation has become an area of research interest. We and others have shown that uniaxial strain promotes smooth muscle differentiation of bone marrow MSCs when cell orientation was restricted by microgrooves.\textsuperscript{24,25} In contrast, for chondrogenesis, the application of cyclic mechanical compression to human bone marrow MSC embedded in biodegradable hyaluronan–gelatin composite scaffolds promotes significant upregulation of chondrogenic markers type II collagen and aggregan.\textsuperscript{26}

Another method of inducing MSC differentiation is using ECMs, which are biological scaffolding proteins that provide structural integrity as well as molecular cues that regulate cell behavior and function.\textsuperscript{27,28} The biophysical and biochemical cues transmitted by the ECM to cells include matrix rigidity, matrix patterning and matrix composition. Physiologically, matrix rigidity varies throughout different tissues of the body, from soft tissues of the brain to hard tissues in the bone. For example, Engler \textit{et al.} showed that physiological rigidity dictated the human bone marrow MSC differentiation.\textsuperscript{29} When cultured on polyacrylamide gels of varying rigidities, the MSCs differentiated to osteogenic lineage on rigid matrix (34 kPa), but soft (0.1–1 kPa) gels promoted neurogenesis. Besides matrix rigidity, the spatial pattern of the ECM also regulates MSC differentiation by restricting cellular shape. For example, small (1024 $\mu$m$^2$) micropatterns of fibronectin stimulated human bone marrow MSCs to differentiate towards adipogenic lineage and retain a rounded morphology, whereas large (10,000 $\mu$m$^2$) islands supported osteogenic differentiation and adherent morphology.\textsuperscript{30} Finally, the matrix composition may also influence MSC differentiation. There is evidence that Matrigel could enhance neuronal differentiation of MSCs.\textsuperscript{31} For example, porcine bone marrow MSCs cultured in three-dimensional patches of polyethylene glycol (PEG)-modified fibrin showed EC phenotype.\textsuperscript{32} These studies suggest that the ECM plays a dynamic role of modulating MSC behavior and phenotype.

Besides soluble, mechanical, and ECM factors in the microenvironment, intracellular factors also play a role in inducing differentiation. MicroRNAs (miRs) are short endogenous nucleotide RNAs that
post-transcriptionally regulate gene expression. In addition to modulating biological processes such as cell proliferation or cell death, they have recently been shown to direct stem cell fate lineage by negatively regulating gene expression.\textsuperscript{33,34} For example, overexpression of miR-21 increased adipogenesis of human ASCs by decreasing TGF-\(\beta\) signaling pathway member TGFBR2 at the mRNA and protein levels.\textsuperscript{35} Conversely, inhibiting miR-21 reduced adipogenesis but led to an increase in TGFBR2 protein levels. Another group demonstrated that miR-148b, miR-27a, and miR-489 modulate osteogenesis in human bone marrow MSCs, even in the absence of osteogenic inducing media.\textsuperscript{36}

Together, these results suggest that MSC and ASC differentiation are controlled by multiple microenvironmental cues and intracellular signaling. Further studies to elucidate the mechanism of microenvironmental factors on differentiation will be critical for directing cell differentiation with high purity for tissue engineering and regeneration purposes.

4. Tissue Engineering and Regeneration Using Bone Marrow MSCs and ASCs

MSCs and ASCs are promising stem cell candidates for the repair or regeneration of diseased tissues, and they have been frequently utilized in \textit{in situ} or \textit{in vitro} tissue engineering approaches to repair various tissues,\textsuperscript{4} including the repair and regeneration of blood vessels, bone, teeth, skeletal muscle, teeth, and spinal cord (Fig. 1). For the \textit{in situ} approach, the cells and other factors are delivered \textit{in vivo}, and the therapeutic potential of the cells is dependent on the interactions of cells and host environment. For the \textit{in vitro} approach, cells and matrix/scaffolds are used to engineer functional tissue constructs prior to implantation. Here we illustrate these different approaches of tissue engineering for the regeneration of the heart, blood vessels, skin and cartilage (Table 1).

4.1 \textit{In situ} tissue regeneration

4.1.1 Cardiac repair

Cardiovascular disease remains as one of the leading causes of mortality in the United States. Over 70 million people in the US are symptomatic from
or at risk of cardiovascular disease.\textsuperscript{37} Stem cell-based approaches to repair or regenerate the heart after myocardial infarction are promising. The goal of stem cell therapy is to regenerate cardiac muscle, enhance angiogenesis and ultimately improve cardiac function. The methods of delivering the therapeutic cells to the heart include systemic delivery, regional coronary infusion, or local myocardial injection.\textsuperscript{38} In one study, autologous swine bone marrow MSC were directly injected into the infarcted heart, and MSCs differentiated towards myogenic lineages as early as two weeks post-injection. Four weeks after cell delivery, the extent of aneurismal thinning and contractile dysfunction were significantly reduced in the cell-treated group.\textsuperscript{39} Besides direct injection, regional and systemic infusion of bone marrow MSCs has also been demonstrated. When $^{99\text{m}}$Tc-labeled rat bone marrow MSCs were transfused to infarcted rat hearts either into the left ventricular cavity or by intravenous delivery, the

\begin{figure}[h]
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\includegraphics[width=\textwidth]{application_of_mscs_for_tissue_regeneration.png}
\caption{Application of MSCs for tissue regeneration.}
\end{figure}
intravenous delivery approach resulted in cell localization predominantly to the lungs and to a lesser degree to the heart.\textsuperscript{40} In contrast, regional delivery produced better retention of cells within the heart, especially in the infarct region, and a lower uptake in the lungs.

Besides delivery of naive cells, MSCs and ASCs have also been genetically modified to improve cell survival or therapeutic effect. In rat models of myocardial infarction, the local injection of rat bone marrow MSCs overexpressing prosurvival factor Akt1 led to reduced intramyocardial inflammation, along with restoration of normal systolic and diastolic function.\textsuperscript{41} In another study, rat bone marrow MSCs that overexpressed anti-apoptotic gene bcl-2 were injected into infarct region. This treatment increased cell survival, decreased infarct scar and promoted neovascularization to assess their survival, engraftment, and functional improvement after myocardial infarction.\textsuperscript{42} These results suggest that genetic modification of MSCs can improve the therapeutic outcome.

\begin{table}[h]
\centering
\caption{Overview of Tissue Engineering and Regeneration Applications Using Bone Marrow MSCs or ASCs.}
\begin{tabular}{lll}
\hline
Organ/Tissue & Treatment & References \\
\hline
Heart & Cell delivery & 40, 41, 63 \\
 & Cell delivery in ECM & 46, 64 \\
Blood vessel & Cell delivery & 47–49 \\
 & Cell delivery in ECM & 22, 50–52 \\
Skeletal muscle & Cell delivery & 65, 66 \\
 & Cell delivery in ECM & 67, 68 \\
Bone & Cell delivery & 69, 70 \\
 & Cell delivery in ECM & 71, 72 \\
Cartilage & Cell delivery & 73, 74 \\
 & Cell delivery in ECM & 61, 62 \\
Skin & Cell delivery & 75, 76 \\
 & Cell delivery in ECM & 55, 56 \\
Teeth & Cell delivery & 77, 78 \\
 & Cell delivery in ECM & 79, 80 \\
Spinal cord & Cell delivery & 81, 82 \\
 & Cell delivery in ECM & 83, 84 \\
\hline
\end{tabular}
\end{table}
Another approach to cardiac repair is to co-inject therapeutic cells with ECMs. ECMs alone such as collagen, fibrin, alginate, and matrigel can enhance neovascularization in the infarcted heart or attenuate infarct scar thinning.\textsuperscript{28,43–45} The rationale behind the co-injection of cells with ECMs is that the ECMs may provide therapeutic enhancement of cardiac function and angiogenesis, while also providing structural support to localize the transplanted cells to the site of delivery. For example, we showed that co-injection of human bone marrow MSCs with fibrin significantly enhanced neovascularure formation following chronic myocardial infarction.\textsuperscript{46}

4.1.2 Peripheral vascular repair

PAD is typically due to atherosclerotic occlusive disease of the peripheral arteries of the limbs, causing symptoms such as intermittent claudication, painful ischemic ulcerations, and limb-threatening gangrene.\textsuperscript{37} A feature of PAD is dysfunction or damage to the vascular endothelium, the diaphanous layer of ECs that exerts control over vascular reactivity, remodeling and angiogenesis. Cell-based approaches to restore or regenerate the endothelium so as to enhance the angiogenic response to ischemia hold promise for the treatment of PAD.

MSCs and ASCs have been examined in the setting of vascular repair for PAD, with the goal of generating and incorporating new functional vessels into existing vessels to remodel them. In one study, mice that underwent hindlimb ischemia, an experimental model of PAD, were treated with adductor muscle injections of murine bone marrow MSCs, mature ECs or culture medium.\textsuperscript{47} The animals treated with MSCs demonstrated significant improvement in limb blood perfusion and attenuation of muscle fibrosis after 21 days, compared to the other treatment groups. The cells did not appear to be incorporated into the mature collateral vessels, but appeared to increase the levels of VEGF and basic fibroblast growth factor (bFGF) in the adductor muscle, suggesting a paracrine mechanism of enhancement. Besides bone marrow MSCs, ASCs have also shown therapeutic benefit for the treatment of hindlimb ischemia. When human ASCs were delivered into immunodeficient mice either at one or seven days after ligation of the femoral artery, the cell-treated animals demonstrated significant improvement in blood perfusion after 21 days.\textsuperscript{48}
In a comparative study of the therapeutic efficacy between human bone marrow MSCs and ASCs, equal numbers of cells were injected intramuscularly into the ischemic limb of immunodeficient mice. Control animals received saline injection only. Two weeks after cell transplantation, laser Doppler blood perfusion imaging showed significantly higher improvement in the ASC-treated group, compared to the bone marrow MSC and control groups. To explore the mechanism of the difference in therapeutic effect, the authors reported that ASCs expressed higher levels of matrix metalloproteinases (MMPs) than bone marrow MSCs. The therapeutic effect of ASCs was mitigated when ASCs were transfected with MMP3 or MMP9 siRNA prior to transplantation into the ischemic limb, suggesting that MMP3 and MMP9 were involved in the proangiogenic effect of ASCs. In summary, these studies demonstrate that MSCs and ASCs significantly improve limb blood perfusion and are promising candidates for treatment of PAD.

4.2 In vitro tissue engineering

4.2.1 Tissue engineering of blood vessels

Bypass surgeries are often used to treat obstructed coronary and peripheral arteries. However, synthetic vascular grafts with small diameter (<6 mm) have high failure rate because of frequent clogging. Consequently, a tissue-engineered vascular graft is a promising solution to this problem. MSCs can differentiate into SMCs and secrete growth factors to recruit ECs, and thus are a potential cell source for constructing vascular grafts. Interestingly, vascular grafts seeded with bone marrow MSCs have improved patency, which is attributed to the antiplatelet adhesion property of heparan sulfate proteoglycans on the surface of bone marrow MSCs.

By engineering biochemical and mechanical factors (i.e. matrix proteins, soluble factors, and cyclic strain) in a bioreactor, the proliferation and differentiation of bone marrow MSCs can be controlled in vascular grafts. In vivo studies have also shown that bone marrow MSCs can differentiate into SMCs and promote endothelialization. However, the electrophysiological profile of bone marrow MSC-differentiated SMCs appeared to be different from that of mature SMCs, suggesting that only
partial differentiation of MSCs into SMCs were achieved. Interestingly, vascular grafts seeded with bone marrow mononuclear cells also have high patency. Whether bone marrow-derived MSCs or stromal cells and ASCs can replace SMCs and ECs as a cell source for vascular graft construction need further investigation.

4.2.2 Tissue engineering of biological skin equivalents for wound repair

Skin wounds result from various conditions, including burns, traumatic accidents, or disease. Standard treatments for wound repair include autologous or cadaveric skin transplantations, but these methods face the potential risks of donor site morbidity and transmission of diseases. An alternative approach is tissue engineered skin equivalents for replacing the damaged skin. Physiologically, skin is comprised of a stratified and keratinized epidermis that physical protects the body, and below the epidermis is the dermal layer that provides strength and support. For the treatment of wound injuries or disease, tissue-engineered skin replacements provide an off-the-shelf alternative that can minimize the potential complications of disease transmission or tissue harvesting. The desirable skin equivalents should quickly adhere to the wound, mimic the physiological function and mechanical properties of normal skin, accelerate wound healing, and resist immune rejection.

Tissue engineering of skin equivalents using bone marrow MSCs and ASCs are promising. In a porcine wound healing model, engineered skin constructs composed of autologous bone marrow MSCs seeded in collagen-glycosaminoglycan polymer scaffolds were grafted onto partial thickness wounds. After four weeks, the wound contraction was measured as the movement of wound edges towards the center of the wound. In comparison to the no graft treatment group, the cell-seeded scaffolds contracted to 57% of the original area and the acellular scaffold group contracted to 51%, which was significantly better than the no treatment control group that contracted to 20% of the original area. Histologically, the MSCs persisted in the epidermis and dermis up to four weeks, suggesting that the cells migrated from the scaffold to the neo-epidermis and dermis. In comparison to the acellular scaffold group after four weeks, the MSC-treated
group showed significant enhancement of vascular density. This data sug-
gested that MSC-seeded biologically scaffolds could reduce wound
contraction and improve neovascularization. In addition to promoting
neovascularization, ASCs also participate in wound healing by differenti-
ating into vascular lineages. Engineered constructs consisting of human
ASCs cultured in decellularized cadaveric dermal matrix were grafted
onto full thickness excisional wounds of immunodeficient mice for up to
four weeks. The results showed that ASCs delivered in the dermal matrix
persisted locally to the site of delivery, improved wound healing, and dif-
ferentiated into endothelial and epidermal lineages. Together, these
studies suggest that engineered skin grafts containing bone marrow MSCs
or ASCs accelerate wound healing, improve neovascularization, and may
differentiate into vascular and epidermal lineages.

4.2.3 Cartilage tissue engineering

Articular cartilage plays an important physiological function of lubricat-
ing between diarthrodial joints and distributing mechanical loads. Due to
the avascular nature of articular cartilage, any damage or disease can be
difficult to heal because of limited regeneration capacity. Current treat-
ments to promote cartilage repair include subchondral abrasion,
microfracture, transplantation of osteochondral plugs and autologous
chondrocyte transplantation, but these approaches do not successfully
restore long lasting healthy cartilage. As a result, tissue engineering is
a promising alternative. MSCs and ASCs are candidate cell sources for
engineered cartilage because of their high expansion ratio and propensity
to differentiate towards chondrogenic lineages.

A number of studies have investigated the role of engineered cartilage
tissue constructs using MSCs or ASCs. In one study, the chondrogenic
potential of human ASCs was tested in three-dimensional ECMs consist-
ing of alginate, agarose or gelatin. In the presence of chondrogenic
media containing TGF-β, the content of cartilage matrix such as hydroxy-
proline and sulfated glycosaminoglycans significantly increased with time
for all three ECMs. To test their in vitro differentiation potential, human
ASC-like cells were grown in alginate gels and transplanted subcuta-
neously into nude mice for up to 20 weeks. Histological analysis
demonstrated that the ASCs formed cartilage and maintained their phenotype without evidence of hypertrophy. Cartilage formation was verified by histological stains for cartilage ECM as well as by gene expression and Western blot characterization for collagen II, SOX9, cartilage oligomeric protein and the cartilage-specific proteoglycan aggrecan.

In addition to synthetic or naturally-derived ECMs for cartilage repair, decellularized cartilage ECM is another potential candidate because it already contains the structural and functional elements of native cartilage. To test the efficacy of decellularized ECM for the repair of cartilage defects, acellular cartilage-derived matrix was generated by decellularizing bovine articular cartilage, grinding the matrix into fine powder, and then remolding the matrix into porous three-dimensional cylindrical constructs.\textsuperscript{61} Rabbit bone marrow MSCs were seeded into the decellularized ECM and then transplanted into full thickness osteochondral defects in rabbits for up to 12 weeks. Based on histological assessment, the cell-treated group had significantly better cartilage quality at 12 weeks, compared to the acellular treatment group. Furthermore, the cell-treated group showed significant improvement at 12 weeks, compared to six weeks, which suggested temporal changes in chondrogenic repair. In a related study using ECMs derived from human cadaveric joints and canine bone marrow MSCs, the cell-seeded constructs were delivered subcutaneously into nude mice for four weeks.\textsuperscript{62} The results demonstrated that the engineered constructs formed ectopic cartilage-like tissue, with evidence of collagen II deposition and positive safranin O staining. In summary, these studies illustrate the potential of cartilage tissue engineering using bone marrow MSCs or ASCs.

5. Future Directions

In summary, bone marrow MSCs and ASCs are candidate cell sources for tissue engineering and regenerative medicine because of their ease and reproducibility of isolation, high proliferation capacity and ability to differentiate into therapeutic cell lineages. However, substantial barriers will need to be overcome before these cells become a standard treatment for clinical care. These challenges include determining the optimal conditions for cell expansion, enhancement in cell viability and function \textit{in vivo}, elucidation
of the mechanisms underlying their repair and regeneration ability, and immune acceptance for allogeneic transplantation. Nevertheless, some clinical trials are already underway (http://www.clinicaltrials.gov). As these limitations become resolved, it is anticipated that bone marrow MSC and ASC therapy will fulfill their promise of clinical efficacy.

Acknowledgments

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1. Introduction

Cell-based therapies are a promising alternative to organ transplantation. Numerous cell sources are under investigation for their efficacy in promoting or contributing to the repair and regeneration of human tissues. Over the past decade, MSCs have attracted much attention in the cell therapy and regenerative medicine fields. Unlike more mature cells, MSCs can be expanded through several passages without loss of differentiation potential, have multi-lineage potential, possess potent anti-inflammatory and immunomodulatory properties, and can be isolated from numerous tissues throughout the body.

While the systemic injection of MSCs has shown some promise as a treatment for immune-mediated disease, their use in tissue regeneration applications has met with little clinical success. Recent work indicates that MSCs fail to engraft long-term, and that any therapeutic benefit
derived from MSCs is through transient tissue residence and secretion of trophic factors. However, many of these studies fail to integrate cellular therapy with tissue engineering approaches for deploying MSCs in the appropriate physiologic context with the relevant microenvironmental and mechanical cues. In this chapter, we will discuss some of the biomaterials that have been utilized to deploy mesenchymal stem cells in tissue repair and regeneration applications.

2. Delivery of MSCs for Repairing Cardiovascular Tissues

Cardiovascular disease is the leading cause of death in the United States, and treatment of vascular disease accounts for a large proportion of health care expenditures. There is a vast shortage of viable cardiac tissues and graft segments available for transplantation due to disease and morbidity. Cell-based approaches to engineering cardiovascular structures represent an exciting alternative to traditional treatment options. In the paragraphs below, we will summarize several recent investigations that have deployed MSCs using various biomaterials to produce new blood vessels and repair damaged myocardium.

2.1 Engineering long-lasting blood vessels

The presence of a robust vasculature is critical to supply nutrients and circulating factors to developing and engineered tissues, as well as those tissues undergoing repair. The feasibility of engineering microvascular networks in vivo has been shown using human umbilical vein endothelial cells (HUVECs), human microvascular endothelial cells (HMVECs), and more recently using cells differentiated from human embryonic stem cells.\(^1\) However, these leaky vessels are rapidly pruned and remodeled without stabilization by pericytes. MSCs are an ideal cell population to promote anastomosis and long-term stability due to their secretion of trophic factors, potential for differentiation toward the myogenic phenotype, and in vivo identity and function as pericytes\(^2\)\(^-\)\(^3\) (Fig. 1). The promise of MSCs has ushered in an exciting era of implanting co-cultures or accessory cells to form new tissues using injectable and implantable materials.
Hydrogels derived from natural or synthetic polymers are a preferred material for implanting cellular populations that facilitate neovascularization due to their mechanical properties and injectable capacity. Hydrogels composed of natural materials are advantageous, as they commonly degrade into easily metabolized degradation products. However, there is significant lot-to-lot variability during their preparation, potentially confounding their widespread use. Moreover, a substantial percentage of the patient population may have established immunogenicity to some animal-derived proteins (e.g. collagen), potentially stimulating an undesirable response to the cell carrier. Synthetic polymers address the limitations associated with immunogenicity, availability, and variability, but may require the incorporation of binding domains to enable cell adhesion or advanced chemistries to attain the desirable degradation parameters. Regardless of the biomaterial, it is critical to utilize a substrate that enables long-term cell survival, remodeling of the gel over time, and the alignment of capillaries to facilitate anastomosis with the host vasculature.

Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma and is rich in extracellular matrix constituents such as laminin, collagen IV, and heparan sulfate proteoglycans. Bone marrow- or cord blood-derived MSCs, together with
bone marrow-derived endothelial progenitor cells (EPCs), were entrapped in Matrigel and implanted into the dorsum of rats. This co-culture yielded an extensive network of human blood vessels after one week and formed functional anastomoses with the existing vasculature. The implanted endothelial progenitor cells were restricted to the luminal aspect of the vessels, while mesenchymal progenitor cells were adjacent to lumens, confirming their role as perivascular cells. Importantly, the engineered vascular networks remained patent at four weeks in vivo. Similar results were found by deploying murine or human MSC-type populations, together with endothelial cells, in fibronectin-type I collagen gel implants. Implantation of the endothelial-mesenchymal co-cultures consistently resulted in stable vessels that persisted in vivo, some for up to one year. However, those implants containing only endothelial cells quickly regressed. The implant material facilitated cell adhesion, proliferation, and arrangement into capillary vessels. Unlike implants formed from Matrigel or collagen that are limited by potential immunogenicity, hydrogels formed of fibrin can be produced from autologous blood samples. Mechanically robust fibrin hydrogels exhibited significant increases in vascular invasion when used to deploy MSCs due to the proteolytic degradation of the matrix by MSC-secreted metalloproteinases. Synthetic polymers have also been used to implant MSCs with an eye toward neovascularization. Differentiated MSCs entrapped within cylinders formed of poly(ethylene glycol) diacrylate enabled robust neovascularization and contributed to the formation of highly vascularized fibrous capsules representative of soft tissue.

Blood vessels must be produced over several length scales, ranging from 3–8 μm capillaries to vascular grafts with diameters exceeding 6 mm. MSCs have been deployed on various biomaterials to assist in the formation of tissue engineered vascular grafts (TEVGs) and support grafts that are endothelialized in vivo. Bone marrow-derived MSCs were embedded within the walls of nanofibrous mesh TEVGs produced from electrospun poly-L-lactic acid (PLLA) by seeding a flat film and rolling it around a mandrel to produce a vessel-like structure (Fig. 2). Grafts implanted for up to 60 days in the common carotid artery of athymic rats facilitated efficient cell recruitment and organization, significant ECM synthesis and self-assembly, and excellent long-term patency. Gong and
Niklason seeded MSCs in mesh scaffolds formed of poly(glycolic acid) (PGA), and cells were cultured under optimized conditions in pulsatile perfusion bioreactors to induce differentiation toward the myogenic phenotype. This graft was readily endothelialized when seeded and maintained in culture over eight weeks. After culture, MSCs expressed smooth muscle cell actin, a hallmark of the myogenic lineage, and MSC-seeded grafts possessed significantly more collagen content and elevated burst pressures compared to earlier protocols. These data suggest that MSCs provide a viable alternative to autologous smooth muscle cells to address the reduced production of vessel-strengthening collagen with age.

Fig. 2. (A) Nanofiber mesh formed by electrospinning of poly(glycolic acid) (image courtesy of Randall Janairo and Song Li, University of California, Berkeley). (B) Fibrin gel observed with confocal reflectance microscopy (image courtesy of Ekaterina Kniazeva and Andrew Putnam, University of Michigan). (C) Polymeric sponge formed by gas foaming/particulate leaching of poly(lactide-co-glycolide).
in TEVGs. Numerous other proteins and synthetic polymers have been electrospun into materials for bioresorbable vascular grafts (reviewed in Ref. 11), but the contribution of MSCs toward vessel formation has not been examined in depth. MSCs have also been entrapped in natural materials and induced toward the myogenic phenotype in culture. Multipotent adult progenitor cells were incorporated in fibrin vascular molds, cultured in the presence of an optimized inductive cocktail for three weeks, and their response to stimulation and mechanical load were characterized.12 Cells within the molds aligned circumferentially as desired, expressed smooth muscle cell-specific genes and surface markers, and generated contractile force when chemically stimulated.

2.2 Implanting MSCs to repair damaged myocardium

Myocardial infarction (MI) and subsequent heart failure represent the main cause of death in industrialized nations. Left untreated, the loss of viable myocardium resulting from MI results in continued expansion of the initial infarct area and left ventricle (LV), finally leading to heart failure. Since cardiomyocytes rarely proliferate or differentiate after injury, transplantation of stem cells is now increasingly recognized as an effective method to repair the infarcted myocardium.13 The mechanism of MSC contribution toward myocardial repair is presently debated in the literature, as is the ideal route and timing of delivery. Intravenous delivery of MSCs is highly inefficient in light of the pulmonary first pass effect,14 thereby necessitating the targeted delivery of these cells. Intramyocardial injection of MSCs has been reported to improve cardiac function and resting blood flow and likely attains higher engraftment efficiency than systemic delivery. However, MSCs are stimulated by the properties of the surrounding diseased myocardium and may still migrate away from the injection site, thereby providing opportunities for instructing the behavior of these cells through materials-based delivery strategies.

Fibrin is a biodegradable, fast gelling matrix, which easily entraps and enables the survival, adhesion, and proliferation of MSCs. The mechanical properties of this biopolymer can be tailored by modulating the fibrinogen concentration and thrombin concentration independently. However, there is a trade-off between mechanical properties and cell
viability, with higher concentrations of each component facilitating rapid gelation of a high-strength product but failing to promote optimal cell viability and growth. To address these limitations, Zhang et al. modified fibrinogen with a benzotriazole carbonate derivative of polyethylene glycol (PEG) to increase the number of crosslinks with adjacent fibrin monomer molecules, thereby increasing mechanical properties while maintaining the viability of encapsulated cells. PEGylation increased the storage modulus by nearly 40% without significantly increasing the gelation time. When MSCs were mixed into the gel, cells proliferated faster than MSCs grown on tissue culture plastic, migrated out of the gel when stimulated, and expressed cell surface markers indicative of differentiation toward the endothelial lineage in the absence of cytokine treatment. Liu et al. entrapped autologous porcine MSCs in a fibrin gel and applied the patch to the LV anterior wall following MI. After 21 days, LV contractile performance was improved in conjunction with increased neovascularization, likely as a function of improved MSC engraftment following migration from the gel and the localized production of trophic factors that stimulate host cell migration into the ischemic site.

MSCs have been implanted on the wall of ischemic myocardium using composite materials designed to capitalize on the benefits of each constituent. Poly-glycolide-co-caprolactone (PGCL) is a synthetic composite material that possesses elasticity, suggesting that it could be employed to engineer a patch for mechanically dynamic environments such as the heart. PGCL scaffolds were seeded with syngeneic bone marrow mononuclear cells and sutured onto the epicardial surface of rats seven days before induction of MI. Animals treated with cell-seeded PGCL patches or PGCL alone exhibited significant reductions in LV remodeling associated with heart failure, while cell-seeded constructs were associated with increased neovascularization. These data suggest that PGCL contributed mechanical integrity to limit LV dilation, while the addition of cells stimulated repair of the damaged myocardium. The potential of MSC-seeded biopolymer composites to promote cardiac repair is still unknown, but this biomaterial represents a promising candidate that possesses elastic material properties and can promote the cardiomyogenic differentiation of MSCs.
3. Delivery Vehicles for Deploying Stem Cells in Skin Regeneration

Chronic skin wounds including those associated with diabetes, affect 5.7 million patients and cost the United States healthcare system an estimated US$20 billion annually. Approximately 40,000 burn patients are admitted to US hospitals every year, and the use of skin grafting to treat burn patients represents a US$1 billion market. Not surprisingly, single modality therapeutic approaches such as growth factor treatment using platelet derived growth factor, have met with little success particularly in light of the complex pathophysiology of chronic and acute dermal wounds. More recently, multifactorial approaches utilizing stem cells and tissue engineering approaches have been applied to treating acute and chronic skin wounds. We will discuss some recent approaches that utilize bioengineered scaffolds to deploy keratinocyte stem cells, fibroblasts, MSCs, and adipose stem cells (ASCs) to restore structure, function, reduce scarring, and improve the cosmetic appearance of skin.

3.1 Cultured epithelial autograft

The use of autograft, allograft, and xenograft skin transplants by Hindus was described in Sanskrit texts and dates to around 3000–2500 B.C. While such approaches are commonly used in current medical practice, extensive burns and diabetes-associated pathology limit the use of autografting in acute and chronic dermal wounds. Allografts can only be used as temporary cover due to immune rejection, and also carry the risk of disease transfer. One of the first cell and tissue engineering approaches developed to overcome some of the difficulties associated with auto and allografting was the development of keratinocyte culture to generate cohesive sheets of stratified epithelium referred to as cultured epithelial autograft (CEA).

Epicel® (Genzyme Biosurgery) is a commercialized CEA product that is generated in two to three weeks using bioreactor technology. Several groups have also evaluated matrices such as fibrin, plasma, collagen, chitosan, hyaluronic acid and polymers such as polyurethane, Teflon® film, and polyhydroxyethyl methacrylate for delivery of pre-confluent
keratinocytes to wound sites. However, CEAs are mechanically fragile, blister, and ulcerate due to the poor formation of a basement membrane and the absence of an underlying dermis. More recent approaches to skin regeneration deploy multiple cell types in more complex, three-dimensional constructs to more closely mimic a complete dermal-epidermal structure for repair and regeneration of deeper wounds.

### 3.2 Dermal substitutes

Dermal substitutes may be cellular or acellular, but lack an epidermal component. As opposed to CEAs, which can take weeks to culture, dermal substitutes are supplied as off-the-shelf products. Dermagraft® (Advanced BioHealing) is a dermal substitute consisting of metabolically active neonatal foreskin fibroblasts on a polyglactin mesh. The foreskin fibroblasts deposit an ECM comprised of collagens, glycosaminoglycans, and growth factors onto the mesh that is then cryopreserved. Dermagraft® has been FDA approved for use in full thickness diabetic foot ulcers. AlloDerm® (LifeCell) is manufactured from donated skin that is decellularized to prevent an immune rejection response, and subsequently freeze-dried. Although it is only FDA approved for breast reconstruction and hernia procedures, it has been used in research settings for treating full thickness dermal wounds.

Several dermal substitutes have also been described in a laboratory setting. In a pig model of dermal regeneration, autologous fibroblasts were deployed in a collagen gel containing alpha elastin hydrolysate. Dermal fibroblasts were seeded onto this scaffold and cultured for ten days. The fibroblasts were applied to a full-thickness wound and covered with a split-skin mesh graft. The scaffolds seeded with the highest number of fibroblasts showed significantly improved healing relative to acellular constructs. In an *in vitro* model, HUVECs were overlayed onto a dermal layer generated by dermal fibroblasts. The dermal layer was shown to support the formation of vessel-like structures by HUVECs in a hepatocyte growth factor (HGF) dependent manner. In a similar model, MSCs were ad-mixed with HUVECs and incorporated into the dermal layer. The MSCs were shown to further stabilize and enhance the formation of these vessel-like structures.
3.3 Bilayered skin substitutes/living skin equivalents

Living skin equivalents (LSEs) approximate the structure of skin, containing both an epidermal and dermal component in which different types of stem cells can be deployed. Similar to dermal equivalents, this category contains an array of tissue engineered constructs for deploying stem cells that are either commercially available or described by individual laboratories. Apligraf® was the first bilayered, allogeneic skin substitute approved for treating chronic wounds.22 Apligraf® is derived from living neonatal foreskin fibroblasts seeded onto bovine type I collagen to generate a dermal layer. Neonatal foreskin keratinocytes are subsequently seeded onto the dermal layer to generate a functional epidermis. In clinical studies evaluating chronic wound healing, patients treated with Apligraf® were more likely to heal faster and more completely relative to the standard of care.22

Integra Bilayer Matrix Wound Dressing and Integra Dermal Regeneration Template are FDA approved for the treatment of chronic wounds and burns, respectively. Both products have a dermal component comprised of cross-linked bovine tendon collagen and shark chondroitin 6-sulfate, and a pseudo-epidermal component comprised of silicon.23 Neither product contains a cellular constituent, a native ECM, or the growth factors present in other bilayered skin substitutes, dermal substitutes, or CEAs. Relative to Alloderm® and Dermagraft®, Integra demonstrates very little construct remodeling.23 The deployment of MSCs within Integra demonstrated significantly increased neovascularization and growth factor incorporation relative to the biomaterial without MSCs.27

Several laboratories have developed alternative delivery vehicles for deploying stem cells in skin regeneration applications. A LSE comprised of autologous keratinocytes and fibroblasts deployed in clotted plasma gel have been successfully used for the treatment of burns. In an in vitro model, ASCs have been used in place of fibroblasts to generate a self-assembled dermal layer in the presence of ascorbate.28 After merging three of these dermal layers, a keratinocyte layer was overlayed to yield a bilayered LSE. ASCs have also been differentiated in vitro to adipocytes and incorporated into a tri-layered LSE with an adipose containing fascia.28
3.4 Other vehicles for deploying stem cells

MSCs deployed in collagen gels generate a dermal-like substitute that is similar to collagen gels containing dermal fibroblasts. Autologous MSCs deployed in fibrin gels were shown to promote healing of large skin wounds associated with skin cancer resection, as well as healing of chronic foot ulcers (duration more than one year) associated with diabetes. In a porcine model of skin wound healing, platelet rich plasma (PRP) has been used as a delivery vehicle for ASCs. In this model, ASCs were shown to increase vessel formation, while PRP with ASCs was shown to improve the cosmetic appearance of the dermal wounds. In a porcine partial-thickness burn model, MSCs were seeded onto freeze-dried collagen-glycosaminoglycan scaffolds and cultured for two days. The skin equivalents were raised to the air-liquid interface for two days to generate an epidermis. Pigs treated with the MSC-seeded constructs demonstrated significantly increased wound contraction, as well as significantly increased vascular density at four weeks.

4. Biomaterials for Implanting MSCs for Regenerating Osteochondral Tissues

MSCs are under intense investigation for use in cell-based approaches to repair skeletal defects. Although these cells can be readily induced to differentiate toward both the chondrogenic and osteoblastic lineages in culture, their direct participation in the formation of each tissue has been limited when delivered to the tissue site. The systemic or local injection of MSCs without an appropriate carrier or vehicle fails to provide the necessary structure and framework for cells to populate tissue defects. As well, many cells migrate away from the defect site or undergo apoptosis or necrosis when placed in the harsh environment. In an effort to improve cell survival and participation in the repair of cartilage and bone defects, extensive efforts are ongoing to develop biomaterials that bridge the defect site and provide a platform for instructing the differentiation of these progenitor cells toward the desired phenotype. We will briefly review recent efforts in the development and application of biomaterials to implant MSCs for the formation and repair of cartilage and bone.
4.1 Deploying MSCs for cartilage formation

Cartilage is an avascular tissue with low cellularity and a limited capacity for self-repair, thereby making it an ideal candidate for cell-based approaches toward tissue engineering. MSCs have generated significant interest in cartilage formation as an alternative to autologous chondrocytes. Chondrogenesis with MSCs generally involves induction with transforming growth factor-β (TGF-β) and a 3D culture environment (e.g. micromass, cell pellet, seeded within biomaterials). With regard to the 3D culture environment, the degree of chondrogenesis is scaffold dependent, thereby providing substantial motivation for careful biomaterial selection for the delivery of MSCs for cartilage repair. The application of biomaterial systems enables more extensive control over cell behavior than the popular cell pellet approach and provides a strategy for limiting cell migration away from the defect site. A host of natural and synthetic materials have been used for MSC chondrogenesis (Table 1), and these materials are broadly divided into substrates that are either injectable or implantable.

<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>Animal Model</th>
<th>Example References</th>
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</thead>
<tbody>
<tr>
<td><strong>Natural polymers</strong></td>
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<td>Alginate</td>
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<td><strong>Synthetic polymers</strong></td>
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<tr>
<td>Poly(α-hydroxy esters)</td>
<td>Mouse, rabbit</td>
<td>48, 53</td>
</tr>
<tr>
<td>Poly(ε-caprolactone)</td>
<td>Swine</td>
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<tr>
<td>Poly(propylene glycol) fumarate</td>
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<tr>
<td><strong>Bioceramics</strong></td>
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<td>Hydroxyapatite</td>
<td>Rat, rabbit, mouse</td>
<td>46, 48</td>
</tr>
<tr>
<td>Tricalcium phosphate</td>
<td>Sheep</td>
<td>38</td>
</tr>
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</table>

Table 1. Commonly Used Biomaterials for Implanting MSCs to Repair Bone and Cartilage.
Hydrogels may be used as injectable scaffolds due to their ability to fill defects of any shape and size. Hydrogels have a high water content, support the transport of nutrients and waste, mimic many characteristics of the extracellular matrix, and have tailorable mechanical properties upon physical or chemical crosslinking. Furthermore, MSCs can be homogeneously suspended within these materials, where encapsulated cells generally retain a rounded morphology that may induce a chondrocytic phenotype. Equine articular cartilage defects of the femoropatellar joint treated with fibrin-encapsulated MSCs exhibited enhanced tissue repair after 30 days characterized by greater tissue formation and increased presence of type II collagen, yet these improvements were lost compared to cell-free fibrin after eight months. Liu et al. reported that MSCs transplanted in hyaluronic acid hydrogels resulted in firm, elastic cartilage that filled the entire defect after 12 weeks, and implanted constructs were well integrated with host cartilage. The encapsulation of MSCs in hydrogels formed of synthetic polymers releasing TGF-β exhibited increased type II collagen distribution and cartilage formation compared to cell-containing gels alone. These findings suggest that chondrogenesis of MSCs in situ may require induction with TGF-β in order to realize the full potential of this approach.

Implantable materials such as sponges and meshes facilitate precise control over material morphological properties compared to hydrogels, and these substrates enable the generation of mechanically stable constructs in vitro for subsequent in vivo implantation. However, these materials suffer from difficulties with filling irregularly shaped defects and maintaining contact along the entire defect periphery, each of which facilitates host cell migration and inhibits the formation of weak fibrous tissue. In light of the responsiveness of MSCs to mechanical cues, whether from the substrate or mechanical loading, the properties of the implant will have a profound contribution toward the resulting phenotype upon implantation. In a recent study, circular poly(ε-caprolactone) (PCL) scaffolds cut from electrospun nanofibrous mats were seeded with MSCs and implanted into full-thickness defects in the femoral condyle of mini-pigs. Six months after implantation, MSC-seeded scaffolds exhibited the most complete repair within the defects compared to materials seeded with autologous chondrocytes or cell-free scaffolds. Similarly, autologous
ove MSCs seeded on \( \beta \)-tricalcium phosphate (\( \beta \)-TCP) implants and implanted into full thickness articular cartilage defects yielded hyaline-like tissue that was indistinguishable from the adjacent normal cartilage.\(^{38}\) Defects treated with cell-free implants contained fiber-like tissue and exhibited incomplete repair after six months.

### 4.2 Deploying MSCs for bone formation and regeneration

Bone autograft has been used for many years as the standard of care for bone regeneration applications such as the repair of non-union fractures, and in ectopic ossification applications such as lumbar fusion. Intrinsic to autograft are the trinity of factors that are critical for optimal bone regeneration: an osteoconductive matrix to allow for new bone and vascular ingrowth, osteoinductive factors to induce new bone formation, and osteogenic cells to deposit and mineralize a bone matrix. The limitations of autograft, namely tissue availability, low fusion rates, and significant morbidity, have motivated the pursuit of alternative materials for bridging bone defects and stimulating bone repair. These materials include allogeneic cancellous bone chips, collagen sponges, synthetic polymer implants, and various hydrogels. Such materials have been described as alternatives to autograft and demonstrate osteoconductive properties, yet for the most part, they lack osteoinductive factors as well as osteogenic cellular components.\(^{39}\) In an effort to provide an osteogenic cellular constituent, several groups have combined stem cells from different sources with these materials. In this section, we will describe the materials that have been used in combination with different stem cells as alternatives to bone autograft in bone regeneration or ectopic/orthotopic ossification applications.

#### 4.2.1 Cancellous bone chips and demineralized bone matrix (DBM)

Bone allograft (cancellous bone chips) and DBM are currently used to treat fractures and in lumbar fusion procedures. They are sold as an off-the-shelf product that possesses osteoinductive activity elicited by endogenous growth factors that remain associated with the ECM. Most
manufacturers offer cancellous bone chips as a decellularized product, but some offer metabolically active cellular cancellous products that are processed and cryopreserved in a manner that maintains the viability of endogenous stem cells (Trinity Evolution, Orthofix). For many years, bone marrow aspirate, containing a heterogeneous mixture of endogenous hematopoietic stem cells, MSCs, and endothelial progenitors, has been used in combination with cancellous bone chips and DBM to generate osteoconductive grafts with augmented osteogenic activity. Cancellous chips loaded with bone marrow were shown to enhance the healing of non-union fractures in the clinical setting. In a posterior segmental lumbar fusion model in the dog, cancellous bone chips in combination with DBM and clotted bone marrow were shown to significantly improve the success rate. Importantly, clotted bone marrow alone demonstrated no beneficial effect.

4.2.2 Collagen sponges and collagen composites

Collagen sponges, such as those found in the INFUSE Bone Graft (Medtronic), and collagen composites, such as Healos (collagen/hydroxyapatite, Depuy), Mozaic, and Vitoss (collagen/βTCP, Integra and Orthovita respectively), are osteoconductive materials that lack both an intrinsic osteoinductive component as well as an osteogenic cellular component. Collagen sponges are highly compressible, while collagen-ceramic composites possess more robust mechanical properties. Both types of scaffolds incorporate the native cell adhesion domains associated with collagen. Recently, stem cells alone or in combination with growth factors have been added to these materials to enhance their performance in bone regeneration and ectopic/orthotopic ossification applications. In a rat model of lumbar fusion, bone marrow aspirate was shown to enhance lumbar fusion mediated by a BMP-2 soaked collagen sponge. By contrast, platelet rich plasma did not augment lumbar fusion when used with the INFUSE kit. In a similar model of lumbar fusion, ASCs transduced with an adenoviral BMP-2 vector and loaded into a collagen sponge demonstrated enhanced lumbar fusion relative to BMP-2 alone. Interestingly, ASCs loaded in a collagen scaffold generated little orthotopic bone and failed to promote lumbar fusion. In an orthotopic,
xenogeneic model of bone formation, undifferentiated ASCs and MSCs implanted in Healos persisted in vivo, while in vitro differentiated ASCs and MSCs did not. In a similar model, in vitro differentiated ASCs loaded into a honeycombed collagen scaffold demonstrated increased bone formation relative to undifferentiated ASCs.

4.2.3 Calcium phosphate and hydroxyapatite ceramics

Calcium phosphate ceramic scaffolds such as Copios bone void filler (calcium phosphate, dibasic, Zimmer) have been used in combination with a number of different stem cell sources to augment new bone formation. While ceramic scaffolds are osteoconductive, they lack osteoinductive factors and do not contain endogenous osteogenic cells. Macroporous biphasic calcium phosphate (MBCP) was used as a scaffold in a rat model of a radiation-induced defect of the long bones. Bone marrow aspirate, MSCs, or ASCs were loaded into these scaffolds and the scaffolds were placed in the defect area. In this model, scaffolds loaded with bone marrow aspirate but not MSCs or ASCs, demonstrated significantly greater bone formation than empty scaffolds, indicating that cultured cells were inferior to fresh isolates. Compared to the low specific surface area (SSA) of synthetic β-TCP, high SSA materials have been developed that demonstrate levels of osteogenic differentiation similar to low SSA materials. Calcium-deficient hydroxyapatite (CDHA) has an SSA of 20–80 m²/g and belongs to the high SSA ceramic group. In a rabbit tibial osteotomy model, CDHA scaffolds loaded with MSC or with PRP demonstrated significantly increased bone volume relative to empty scaffolds. There was no added benefit when MSC and PRP were combined, suggesting that CDHA is enhancing bone formation through stimulating MSC differentiation as a function of substrate rigidity or composition.

4.2.4 PLGA and PCL based scaffolds

Similar to ceramic scaffolds, PLGA and PCL based scaffolds are osteoconductive but do not have any osteoinductive properties. Both PLGA and PCL have good biocompatibility profiles and biodegrade at rate that is dependent on their composition. Different stem cell populations have
been seeded on these scaffolds to provide an osteogenic component for bone regeneration applications. In a rabbit critical-sized femoral defect model, porous PLGA scaffolds were loaded with the osteoinductive factor dihydroxy vitamin D3 (Calcitriol), MSCs, or Calcitriol with MSCs and placed in the defect. Only scaffolds containing Calcitriol showed a bony union at nine weeks. Scaffolds incorporating MSCs had greater amounts of pre-mineralized matrix (osteoid) present, but Calcitriol was required for successful union. In a mouse ectopic bone formation model, ASCs were loaded in a PLGA/hydroxyapatite composite in the presence or absence of BMP-2 and implanted subcutaneously. Scaffolds with ASCs alone had negligible amounts of mineral, while scaffolds loaded with BMP-2 alone had significant amounts of mineral. ASC loaded scaffolds with BMP-2 demonstrated the greatest amount of bone formation. In a similar model using porous, honeycombed PCL-TCP composite scaffolds, the osteogenic capacity of fetal bone marrow MSCs, umbilical cord MSCs (UC-MSCs), adult MSCs, and ASCs was compared. The stem cells were loaded on scaffolds, differentiated with dexamethasone and ascorbate in vitro, and subsequently implanted. Scaffolds loaded with fetal MSCs and adult MSCs demonstrated the highest levels of bone formation, while adipose MSC loaded scaffolds demonstrated the least.

4.2.5 Hydrogels

Hydrogels are appealing candidates as scaffolds given their similarity to native ECMs and high degree of biocompatibility. Moreover, these materials enable highly efficient incorporation of cells and inductive factors to stimulate bone formation. In a rat critical-sized cranial defect model, ASCs alone, or ASCs genetically modified with an adenovirus to overexpress BMP-2 were loaded into an alginate hydrogel and implanted into the defect. Only scaffolds with BMP-2 expressing ASCs demonstrated closure of the critical-sized defect at 16 weeks. In a rat calvarial defect model, BMP-2, MSCs, or MSCs with BMP-2 were loaded into hyaluronic acid based hydrogels and implanted in the defect. While MSCs demonstrated poor adhesion to the scaffolds in vitro, scaffolds seeded with MSCs and BMP-2 demonstrated robust bone formation four weeks after implantation. Scaffolds seeded with MSCs alone showed slightly less bone formation.
formation, while scaffolds seeded with BMP-2 alone showed the least amount of bone formation. In an ectopic bone formation model, an MPEG-PCL in situ forming gel was used as a scaffold for implanting ASCs. Only scaffolds loaded with ASCs demonstrated in vivo bone formation, while scaffolds loaded with ASCs and an osteogenic supplement had the highest levels of bone formation.

5. Conclusions

MSCs have tremendous potential to contribute to the repair of human tissues. In order to capitalize on the promise of these multipotent cells to differentiate to many phenotypes, secrete tissue-inducing factors, and suppress inflammatory responses, new approaches for deploying these cells must be developed and optimized under conditions that approximate human physiology. Furthermore, the selection of the delivery vehicle enables control over the mechanical properties, degradation time, cell distribution, cytokine, growth factor, and morphogen gradients, and can even instruct cell fate. The examination of these systems under physiological conditions and in clinically relevant animal models will increase our understanding of how MSCs contribute to the development and repair of human tissues. Importantly, the results of these studies will provide new information on the efficacy of these materials to support the long-term survival and performance of MSCs in tissue repair and regeneration.

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1. Cell Therapies for Myocardial Infarction and Heart Failure

As a leading cause of death in the United States, congestive heart failure (CHF) post-myocardial infarction (MI) has incited the need to develop novel techniques that prevent muscle wall scarring and ventricular dilation, symptoms which contribute to impaired heart function. CHF is typically induced from a MI in which a major coronary vessel becomes occluded, preventing appropriate oxygen exchange in the muscle downstream of this blockage. Ensuing cell death in the myocardium is counterbalanced by increased secretion of collagen, which forms a fibrotic scar to maintain wall integrity. As a consequence of this, ventricle contraction is impaired and results in negative left ventricular (LV) remodeling and dilation of the heart.\(^1\) CHF-induced impairment results in a high morbidity rate as the body has limited capacity to replace damaged tissue and restore function. Although recent studies have reported on the ability of the heart to repair itself after injury via progenitor cardiac stem cells (CSCs) and homing of hematopoietic stem cells (HSCs),\(^2\,\,^4\) intrinsic cardiac regeneration is limited by extremely slow cell turnover in the
myocardium, and turnover only further slows in older patients where CHF predominates. Over the past two decades, novel strategies have taken many routes and used many cell types to treat MI and CHF. Here we present a succinct review of three common regenerative medicine and tissue engineering strategies: cellular cardiomyoplasty, cardiac patches, and injectable scaffolds (Fig. 1). Though numerous variations have been

Fig 1. Approaches to cell-based therapy in the heart. Following a myocardial infarction, cells in liquid solutions of saline or cell culture medium (cellular cardiomyoplasty), and in combination with a biomaterial scaffold (cardiac patch and injectable scaffold) have been explored to prevent progressive left ventricular remodeling that leads to left ventricular dilation and heart failure. Each technique has its own pros and cons as listed here, yet all approaches have had some success in animal models.
attempted for these techniques, we will limit our discussion to the clinically-relevant cell types used, their mechanistic characterization in vitro, and subsequent in vivo results.

Before presenting these methodologies however, it is perhaps helpful to categorize the clinically used cell types today. Since its introduction in the 1990s, cellular cardiomyoplasty, where somatic cells are injected in liquid solutions of saline or cell culture medium into the post-MI heart wall, has gained much attention as one of the first cell-based methods proposed for repairing, replacing and restoring function to the injured heart. Early studies in animal infarct models showed implanted somatic cells survive, prevent further ventricular dilation, and confer some level of improved myocardial output.6–8 Despite some promising results in animal models, clinical trials with autologous somatic cells using various injection methods, while not showing adverse patient effects, have produced mixed results for improvement of global contractility,9–11 with the mechanisms behind improved cardiac function in these studies remaining unknown. One significant shift in the technique to better determine its efficacy has been to use adult stem cells as they are regarded as the optimal source since they are easily obtained, readily expanded in vitro, non-immunogenic, and can be an autologous cell source.

Hematopoietic stem cells (HSCs) are bone marrow-derived cells that can regenerate myeloid and lymphoid lineage cells of the blood, though there is some evidence that these cells are multipotent and can become non-blood derived lineages.12 Their ability to differentiate into cardiomyocytes in vitro and in animal models with infarcted myocardium, however, has proven less than successful.12–14 Mesenchymal stem cells (MSCs), named after their ability to differentiate into mesodermal lineages such as adipose, muscle, bone, etc.15 are also multipotent stem cells which can be easily isolated from the bone marrow where they are relatively abundant.16 These cells lack several cell-surface markers enabling them to be immune privileged,17 and in culture, these cells have been shown to differentiate into beating cardiomyocytes by the addition of the demethylating agent, 5-azacytidine.18 It should be noted that though cells produced by this method exhibit some genotypic and phenotypic characteristics of adult cardiomyocytes, they lack the presence of contractile elements.
With this supporting evidence, human MSCs were once thought to be the most clinically relevant source for myocardial cell therapy. Some studies have reported the ability of MSCs to marginally restore function and structure to animal infarct models by differentiating into cardiomyocytes and inducing angiogenesis though it was later demonstrated that improved cardiac function was likely due to paracrine effects of the transplanted cells and not functional restoration. Others suggest that implanted MSCs fused with resident cardiomyocytes and thus do not actually differentiate into functioning cardiac muscle cells. Increasing infarct cellularity, which increases wall thickness and thus reduces wall stress, is also postulated to result in the observed functional effects. Although the mechanisms underlying improvements in heart function have yet to be identified, the results of cellular cardiomyoplasty in animal models have prompted several human clinical trials. Both skeletal myoblasts and human MSCs were injected into the myocardium post-MI, and little to no improvement was observed. Thus it appears that injection of cells alone into injured myocardium may not be sufficient to restore function and structure due to some mechanism blocking their regenerative capacity. In fact, recent evidence which we will discuss next appears to indicate that MSCs in the infarct differentiate not into muscle but into cells that form small calcified lesions, typical of MSC-derived osteoblasts (see Fig. 2C).


Cells are highly responsive to their surroundings, and when presented with a disease microenvironment, their function is likely different from what is observed in a healthy setting. In healthy tissue, forces, integrins, and the surrounding fibrous scaffold called the extracellular matrix (ECM) exist in a balance termed “dynamic reciprocity.” Thus altered cells programmed by this damaged environment may not be able to sufficiently regenerate tissue function or may even worsen the cell niche. The inconclusive results from MSCs used in this treatment could be explained in part by improper epigenetic regulation as dictated by the aberrant surrounding ECM. Indeed as cells necrose in the heart wall, fibroblasts
Cellular cardiomyoplasty. (A) MSCs grown on hydrogels of defined stiffness develop cell morphologies reflective of neural, myocyte, and osteocyte lineages. (B) MSCs were injected into infarcted hearts (left; with inner ischemic and outer border zones highlighted in shades of blue) where the elasticity of the wall was significantly changed downstream of the occlusion site as shown in the right panel using atomic force microscopy. (C) Histological analysis of the heart wall shows that 29 days after injecting $10^5$ MSCs, hearts showed massive calcifications (black deposits; left image) that originated from injected EGFP+ MSCs.
secrete a substantial amount of collagen to form a scar in the infarct area. This is initially a compensatory mechanism to resist wall deformation and rupture.\(^{28}\) However, as noted by Berry and co-workers, this fibrotic scar is three- to four-fold stiffer than normal muscle, as measured using an atomic force microscope (AFM),\(^ {29}\) and is accompanied by a variety of ventricular remodeling.

Resistance to deformation from a force, i.e. the “stiffness” or more formally the elasticity (\(E\), measured in Pascals, Pa), provided by ECM for cells to contract against, has an important role in development and function. Tissue-specific matrices have distinct mechanical properties that help to direct developing cells to a specific lineage.\(^ {15}\) It has been demonstrated that MSCs plated onto collagen I-coated polyacrylamide (PA) gels that mimic the elasticity of the brain (0.1 – 1 kPa), muscle (~ 8–17 kPa), and bone (<30 kPa), give rise to neurocytes, myocytes, and osteocytes, respectively\(^ {30,31}\) (Fig. 2A). Such responses are also seen with fibronectin but not with laminin-1 and collagen IV-coated gels,\(^ {31}\) suggesting that cells are highly responsive to their physical environment when ligating appropriate integrins. If placed into an abnormally rigid environment in vivo, e.g. one that is three- to four-fold more stiff than normal myocardium and full of collagen I,\(^ {29}\) such results would imply that MSCs would respond by becoming osteoblast-like rather than muscle-like (Fig. 2B). Though there are a variety of soluble factors that may attenuate this process, much of that is likely absent in this infarcted niche.

Such observations also highlight the glaring differences between the in vivo environment, in vitro mimics (e.g. PA gels), and traditional myocyte cultures from the past several decades, i.e. cells grown on collagen-coated rigid glass and tissue culture polystyrene. For the rigid substrate, three-step myofibrillogenesis\(^ {32}\) is halted at the initial pre-myofibril step in isolated myotubes as shown by Griffin and co-workers;\(^ {33}\) instead of mature fibrils, they find that large stress fibers appear and myotubes are overly adherent to the rigid substrate.\(^ {33}\) Conversely, many labs have studied myofibrillogenesis in culture, with cells grown either (1) in syncytia, where clustered cells can contract against one another rather than the rigid matrix, or (2) on glass coated with thicker collagen layers, effectively making the surface more compliant. For example, when cultured on collagen gel-coated glass, with elasticity closer to muscle than rigid
substrates, precardiac mesoderm was able to undergo myofibrillogenesis and could beat rhythmically in similar collagen gels. Detailed analysis of cardiomyocyte behavior as a function of matrix stiffness has shown that mature and neonatal myocytes require a compliant niche that is as soft or softer than healthy muscle for sustained contraction and proper expression of sarcoplasmic/endoplasmic reticular calcium ATPase, calcium storage and transients, and traction stresses. Yet until now the relatively low rigidity of the in vivo environment for the myocardium has largely been underappreciated.

When returning to the infarct example, it is thus not surprising that somatic cells as well as stem cells are regulated by the mechanical context of their environment. While the highly ischemic region in the center of the infarct may lack sufficient blood supply for a cell therapy alone to work, stiffness within the borderzone, which is only 50%–200% higher than normal, likely prevents MSCs from restoring function there as well since no MSC-derived cardiomyocytes were found. As illustrated in Fig. 2, MSC-treated infarcted hearts sometimes show limited recovery and abnormal differentiation, agreeing with in vitro data demonstrating that MSCs become osteogenic on gels with $E \approx 30–50$ kPa. Surprisingly, osteogenic lesions are found throughout the infarct, indicating that at least modest cell engraftment occurred throughout a dominant fraction of both the highly ischemic necrotic core and the modestly ischemic borderzone. However, clinical studies using both similar marrow-derived stem cells, presumably with the same abnormal differentiation characteristics, and other somatic cells appear to demonstrate that there is still some improvement in global function as cells either are directly injected or home to the injury site; observed improvement includes 6%–9% increase in LV ejection fraction, reduced end-systolic LV volumes, and enhanced perfusion in the infarcted area four to six months after cell transplantation. Such discrepancies between studies directly measuring elasticity and implicating problems with differentiation versus those that demonstrate modest functional improvement may likely be the result of differences in variable such as time-post infarct for cell injection, injection cell number, cell preparation protocols, spatial distribution of injections, etc. With differences in time-post infarct for cell injection in particular, cells seeing a pre-formed rigid scar versus a matrix that is actively remodeling could
present environments that are either already rigid or one that is still soft but actively remodeling, respectively. Cells injected into the latter scenario may have a better chance of remodeling the environment, but a more detailed examination is likely required.

Though more appropriate cell types for this therapy may exist, such as more recently discovered cardiac stem cells, these data imply that it is not possible to rely solely on direct injection of cells to remodel the matrix as it provides too few cues in the proper direction to promote the formation of appropriate cardiomyocyte lineages. Instead, two alternate approaches have been proposed which involve delivering cells in epicardial sheets or in injectable scaffolds.

3. Tissue Engineering Approach: Utilizing Biomaterial Scaffolds

While there has been some success in animal models with cellular cardiomyoplasty, and numerous clinical trials are currently ongoing, this technique is plagued by limited cell retention and transplant survival. Injection into an ischemic environment and anoikis (cell death from the absence of cell-matrix interactions) are two major factors attributed to poor cell transplant survival. To overcome both of these challenges, tissue engineering approaches are currently being examined. In this case, cells are given a temporary extracellular matrix through the use of a biomaterial scaffold, unlike the typical cellular cardiomyoplasty approach where cells are delivered in only a liquid solution. Biomaterial approaches also have potential for growth factor delivery, which can further promote cell survival and reduce the ischemic environment.

3.1 Cardiac patches

Myocardial tissue engineering can be categorized into in vitro engineering and injectable approaches (Fig. 1). In vitro engineered myocardial tissue, involving the classical tissue engineering approach of seeding cells on a scaffold and culturing the construct prior to implantation, was the first to be examined in the myocardium. In addition to utilizing pre-formed scaffolds, soluble scaffolds have been pre-mixed with cells and
shaped into the appropriate construct. Moreover, temperature responsive polymer coated substrates have been employed to generate cell monolayers or sheets, which retain their secreted matrices. These can be subsequently combined to form a cardiac patch over the infarcted region. By providing an extracellular matrix for transplanted cells, cardiac patches have been shown to increase cell survival, induce neovascularization, attenuate negative LV remodeling, and preserve cardiac function in pre-clinical models. While improvements in contractility are theoretically possible with stem cell-derived cardiomyocytes, such effects to date are likely through paracrine mechanisms.

Clinical results with this approach are sparse to date, yet results from a non-randomized Phase I clinical trial with a cell seeded collagen scaffold are encouraging at least in terms of safety and feasibility. Mononuclear bone marrow cells isolated from the patient were seeded on a porous collagen matrix ($7 \times 5 \times 0.6$ cm) in the operating room and then sutured onto the epicardium following a single off-pump coronary artery bypass graft surgery (OP-CABG). In chronic infarcts, with an average age of approximately eight months, this cardiac patch increased wall thickness, limited negative LV remodeling, and improved diastolic function; however, patients also received injections of the bone marrow cells in autologous serum into the infarct and borderzone.

While in vitro engineering of a patch to cover up damaged myocardial tissue is a viable strategy for cardiac repair, there are limitations with this approach. For instance, as with other vascularized tissues, the development of sizable constructs in vitro is a major challenge. Secondly, these patches can only be applied to the epicardial surface and thus do not directly treat the infarct. Implantation of a patch would also require an invasive surgical procedure, unlike injectable, percutaneous approaches. However, there are some distinct advantages that this in vitro approach offers. For instance, organization and alignment of transplanted cells can be uniquely achieved. While this is likely not critical for cells that are functioning strictly through paracrine effects, it would be important for cell types that can undergo cardiomyogenesis. New approaches to cardiac patch design include the combination of prosurvival and angiogenic growth factors, and pre-vascularization on the omentum, which is a blood-vessel enriched membrane. This approach improved subsequent patch
integration with host myocardium, leading to preservation of LV volume and ejection fraction, and has potential for the use with other cell types. A caveat however with this study was that LV volume and cardiac function were not statistically different than acellular patches also pre-vascularized on the omentum. More sophisticated biomimetic scaffolds are also providing potential advances in the field. Protocols for decellularizing myocardium\textsuperscript{51,52} have opened up the possibility of creating a cardiac patch with a scaffold that contains many of the biochemical and mechanical cues that match original myocardial ECM composition and potentially its mechanical properties, e.g. 10–20 kPa,\textsuperscript{29} as well. To that end, synthetic scaffolds have also been engineered to match the anisotropic properties of healthy myocardium, albeit only right ventricular myocardium has been successfully mimicked to date.\textsuperscript{53}

### 3.2 Injectable scaffolds

Given the push towards more minimally invasive surgeries that require less recovery time and reduce the chances of infection, injectable approaches to myocardial tissue engineering, which could be delivered minimally invasively through a catheter, are particularly attractive. Moreover, an injectable therapy could be delivered throughout the infarct wall and borderzone, and not solely to the epicardium as with cardiac patches.

Injectable scaffolds for \textit{in situ} myocardial tissue engineering can be utilized as either acellular or cellular treatments.\textsuperscript{42} In the first approach, material is injected into the infarct alone, and can serve to increase cell migration into the infarct area, including neovascularization, to thicken and support the LV wall, or both. As cell therapy, injectable scaffolds can be employed to increase cell transplant survival over the typical cellular cardiomyoplasty approach. An acellular approach may reach the clinic sooner since it has the potential to be an off-the-shelf treatment without the added complications that cells bring, including the appropriate source, the need for \textit{in vitro} expansion, or potential disease transmission. However, cellular therapies are currently in clinical trials, despite poor cell survival as with the typical cellular cardiomyoplasty technique. Injectable scaffolds have the potential to increase cell transplant survival,
with potential functional benefit, and as such are often viewed as an improvement over previously discussed methods.

With the knowledge of these pros and cons, it is critical to define design criteria associated with an ideal injectable scaffold for cell delivery in the myocardium. First, an injectable myocardial scaffold should promote neovascularization to reduce the ischemic environment, promote cell adhesion, survival, and maturation in the case of progenitor or stem cell delivery, and be injectable through a catheter. In terms of cell adhesion and survival, material choice and composition are especially important and should likely mimic both biochemical composition, which includes a complex mixture of proteins and polysaccharides, and mechanical properties of the native myocardial ECM which they are attempting to replace. To date, several materials have been examined for injectable approaches to myocardial tissue engineering, including fibrin,\textsuperscript{54,55} collagen,\textsuperscript{56} Matrigel,\textsuperscript{57–59} alginate,\textsuperscript{60,61} and self-assembling peptides\textsuperscript{42} among others. Collagen has been utilized to promote neovascularization,\textsuperscript{56} thicken the infarct wall and improve LV geometry,\textsuperscript{62} and deliver cells into the myocardium,\textsuperscript{63} however, neutralized collagen gels rapidly at even room temperature. Matrigel, both alone\textsuperscript{57,58} and in combination with collagen,\textsuperscript{59} has been shown to facilitate cell transplantation. Yet, clinical translation of Matrigel is very unlikely since it is a matrix derived from mouse sarcoma, and is known to promote tumor growth \textit{in vitro} and \textit{in vivo}. Alginate, which is a polysaccharide derived from seaweed, has been utilized mostly for thickening and supporting the LV wall,\textsuperscript{60,61} however, as a regenerative scaffold, alginate is likely not the best choice since it is known to have poor cell adhesion,\textsuperscript{64} although it can be modified to contain cell adhesion peptides.\textsuperscript{65} Self-assembling peptides can form nanofibrous networks inside the myocardium, which promote cell and vessel ingrowth.\textsuperscript{66} Yet, these matrices were not capable of improving cell transplant survival.\textsuperscript{67} Of all of these materials, the only neutralized material to be injected via catheter is alginate, which is being explored as an acellular therapy.\textsuperscript{68} Thus, while a material may be injectable via a syringe, this does not necessarily translate to percutaneous, minimally-invasive delivery. In fact, despite increased survival in small animal models with injectable scaffolds, no materials have advanced into clinical trials for enhancing cell therapy in the myocardium.
The ECM is known to play a role in almost every cell process including attachment, differentiation, morphogenesis, and whether a cell proliferates or apoptoses, and thus, the typical goal of tissue engineering scaffolds is to mimic the in vivo ECM for a particular tissue, mainly because of the importance of cell-matrix interactions. Recently, an injectable, in situ gelling scaffold derived from decellularized ventricular myocardium was tested in vivo, which retains many of the original biochemical cues of native cardiac ECM. This material has also been recently injected via an endocardial, percutaneous approach in a porcine model, which is the preferred delivery method for cell therapy in the myocardium. While the biochemical aspects of the myocardial ECM have been effectively mimicked, no injectable materials have been designed to mimic the mechanical properties, whose importance has been highlighted above and likely plays an important role in determining transplanted cell fate.

3.3 Material selection

Considering the key role of cell-ECM interactions in affecting cell behavior, and the tissue specificity of the ECM, a scaffold that possesses the appropriate ventricular ECM cues would be ideal for myocardial tissue engineering. However, few materials for cardiac tissue engineering have involved this design approach. Neither naturally derived or synthetic materials possess all of the necessary attributes for a scaffold, whether for a cardiac patch or an injectable scaffold; each has their pros and cons.

Protein and natural-based hydrogels are especially applicable in tissue engineering strategies due to their ability to better mimic the in vivo microenvironment by exhibiting familiar ECM components and structural organization. Cells can thus better respond to the signals provided by familiar components in their surrounding matrix. Additionally, these molecules are generally biocompatible, thereby reducing concerns over potential toxicity. Synthetic biomaterials on the other hand can provide better mechanical properties and can be more easily manipulated than natural polymers.

Currently no ideal materials exist in terms of mimicking both biochemical and mechanical properties of the myocardium. Advancement in
this field will likely be achieved by the rational design of materials to appropriately mimic the desired properties of the native ECM. This will likely change depending on the intended mechanism of therapy: structural vs. paracrine vs. regeneration. Furthermore, designing materials with clinical translation in mind is a necessity. For example, although a material may be injectable via syringe, it does not necessarily translate to a minimally invasive approach. Design criteria must therefore include translation to man from the very beginning with small animal studies.

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1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in the world. Ischemic heart disease, cerebrovascular disease and peripheral vascular disease account for 13 million deaths annually. Additionally congenital heart disease, which represents the most common birth defect affecting nearly 1% of all live births, remains a leading cause of death in the newborn period. In the United States alone, the American Heart Association estimates the cost of treating CVD in 2009 to be US$475.3 billion.

For end-stage cardiovascular disease in adults, or in children with congenital cardiovascular anomalies, surgical reconstruction remains the treatment of choice. Unfortunately there is a relative paucity of autologous tissue available for reconstructive surgeries, necessitating the use of synthetic materials.

Biomaterials available in the cardiovascular surgeon’s armamentarium include autologous vessels and prosthetic materials. Autologous tissue is superior to prosthetic materials such as Goretex®, as prosthetics are more
prone to thrombosis, infectious complications, neointimal hyperplasia, and accelerated atherosclerosis. In an adult patient undergoing multiple cardiac or peripheral vascular procedures, however, suitable autologous tissue may be scarce due to the diffuse nature of atherosclerosis. Additionally, in children undergoing surgery for complex congenital heart defects, repairs may be extra-anatomic and require more autologous tissue than is available. Clearly, there is demand for improved, biocompatible vascular conduits in these populations.

The great promise and hope of vascular tissue engineering is the development of biological substitutes that restore, maintain or improve tissue function. The origins of vascular tissue engineering can be traced back to Alexis Carrel who received the Nobel Prize in medicine for his early work in vascular biology. This established the basic principles that provided the foundation for vascular surgery. Later in his career while working in collaboration with Charles Lindbergh at the Rockefeller Institute, some of the early pioneering work in cell culture was performed. In their manuscript entitled “The Culture of Whole Organs,” Carrel and Lindbergh postulated that cell culture would one day be used to grow entire organs. However it was not until Weinberg and Bell’s seminal paper in 1986 that Lindberg and Carrel’s prediction became a reality for vascular tissue. This search led to many advances in our basic understanding of tissue interactions, including the critical importance of cellular and matrix interactions, the modulation of cellular recruitment, growth and differentiation, as well as a deeper appreciation for the challenges that are faced in attempting to apply these discoveries clinically. In this chapter we aim to guide the reader through the major milestones of vascular tissue engineering while highlighting the importance of continued scientific investigations aimed at reducing the tremendous burden of cardiovascular disease.

1.1 Historical overview

The first report of the use of synthetic vascular grafts dates back to 1952 when Voorhees et al., used Vinyon N cloth tubes as arterial interpositions in dogs. Prior to this report, scientists were focused on using native arteries as conduits. In the ensuing years, other synthetic materials aimed at passively transporting blood with minimal reaction were
developed, leading to the clinical success of polyethylene terephthalate (Dacron) and expanded polytetrafluoroethylene (ePTFE).

Major achievements in creating an ideal conduit have driven the continued search for the best strategy to create fully functioning blood vessel substitutes. In 1986, Weinberg and Bell’s landmark publication described the use of bovine cells with rat collagen gel to create an artificial blood vessel model. In 1998, Shinoka et al., first described the use of a biodegradable synthetic scaffold with ovine cells, demonstrating long-term autologous implantation in a low pressure pulmonary artery system. Also in 1998, L’Heureux et al., published their creation of an engineered graft that used human cells and was tested as a short-term xenogeneic implant in a high pressure arterial bypass model. In 1999, Nicklason et al. and Shum-Tim et al. described the creation of an artery grown in-vitro using autologous porcine/ovine cells on a biodegradable polymer. In their studies, Niklason et al. used small diameter grafts, and studied their vessels under arterial pressures in the short-term. Shum-Tim et al., developed large diameter grafts, studied under lower pressures with long-term evaluation of function.

In 2001, Shin’oka et al., reported the first clinical use of a tissue-engineered vascular construct. The vascular graft was implanted in a four-year-old girl to reconstruct an occluded pulmonary artery, after a prior Fontan procedure. In another major milestone for vascular tissue engineering, in 2007 L’Heureux et al. used their sheet-based tissue engineered blood vessel clinically as replacements for failing arteriovenous shunts. Despite these tremendous achievements, the field has yet to report the clinical use of a tissue-engineered vascular construct as a fully arterial interposition in humans. Further, the mainstay therapy for vascular reconstruction continues to be autologous arteries and veins. In the following pages we will detail these early successes, discuss the applications of vascular tissue engineering in clinical trials, and describe the frontiers of our technology.

2. Critical Elements of an Artificial Blood Vessel

Synthetic vascular grafts were developed for patients with insufficient autologous tissue for bypass grafting. These grafts have reasonable safety
profiles, satisfactory surgical handling characteristics (i.e. suture retention), and are available “off-the-shelf” for use as large caliber bypass grafts. Currently available synthetic vascular conduits, however, have several significant limitations. These grafts have no growth potential, thus many pediatric patients will “outgrow” the graft and require re-operation. Re-do operations are associated with an increased risk of complications and death. Bioprosthetic materials such as gluteraldehyde-fixed xeno- or allo-grafts used for grafting are prone to ectopic calcification, resulting in poor durability. Both prosthetic and bioprosthetic materials are prone to infection, putting the patient at risk for sepsis, graft rupture, distal septic emboli, as well as re-operation for explantation of graft material. Lastly, the use of synthetic grafts as small caliber bypass grafts is limited as they are prone to thrombosis and neointimal hyperplasia, likely from a lack of biocompatibility and an inability to repair and remodel.

Tissue-engineered vascular conduits address the shortcomings of currently available vascular conduits. The ideal tissue-engineered graft possesses excellent surgical handling characteristics. An experienced surgeon must be able to handle the graft, modify it as necessary for the patient’s anatomy, perform anastomoses using standard surgical technique and instrumentation, and obtain hemostasis immediately following implantation. Because of the morbidity and mortality associated with longer operative and anesthetic time, the surgeon must perform these anastomoses in a timely fashion. Optimally, tissue-engineered grafts would be available “off-the shelf” (similar to prosthetic grafts), and require minimal manipulation other than seeding the cell on the substrate the day of surgery.

In addition to satisfactory surgical handling techniques, the ideal tissue-engineered vascular graft is biocompatible. The polymerized scaffold or decellularized matrix should degrade over time, leaving intact vascular neotissue that provides the structural integrity for the conduit. The degradation of scaffold materials results in a completely biocompatible structure that is not prone to infection or ectopic calcification, and does not require immunosuppression. Finally, the ideal tissue-engineered graft possesses the intrinsic ability to grow with the patient, obviating the need for re-operations in the pediatric population.

The most important characteristic of a tissue-engineered vascular graft is, however, recapitulation of vessel form and function. Put simply, grafts
implanted in the arterial and venous circulation should mirror as closely as possible the native artery and vein. The ideal arterial interposition graft possess a functional, confluent, non-thrombogenic endothelium and a thick smooth muscle laden tunica media that can accommodate mean arterial pressure and constrict appropriately to ensure perfusion to end-organs during low flow states. Thus, the intrinsic mechanical strength of the seeded scaffold should be higher and the degradation time longer in arterial grafts than in venous grafts where mechanical integrity is less critical but adequate compliance is necessary.

3. Approaches to Creating TEBVS

3.1 The first artificial artery: Weinberg and Bell

The first reported successful construction of a tissue-engineered vascular graft came from Weinberg and Bell in 1986. Prior work had established the use of synthetic materials as vascular conduits, albeit not for small diameter vessels (< 6 mm diameter), and autologous tissue had become the mainstay of vascular repair. Building on previous reports of partial vascular constructs, including in vitro growth of endothelial cells and models of the vascular wall in mock circulatory loops, this seminal paper reported culturing bovine vascular cells to seed a collagen matrix in an tubular mold.8,19

Specifically, bovine smooth muscle cells were combined with collagen using a casting culture medium to create a tubular lattice. After a week of culture, a synthetic Dacron sleeve was placed on the outer surface of the construct, and seeded with fibroblasts to create a neo-adventitia. After another two weeks, the grafts were seeded with endothelial cells and left in a rotational culture (1 rev/min) for one week. This procedure provided a template for the creation of a tubular cellular structure that demonstrated extracellular matrix deposition of collagen, alignment of smooth muscle cells and a confluent endothelial cell layer, in a construct that had burst pressures of up to 323 mmHg when reinforced with added layers of Dacron. This led the authors to describe the use of their graft as a model for the study of the biological properties of blood vessels, rather than as a potentially clinically useful construct. Despite these shortcomings, Weinberg and Bell laid the foundation and provided a roadmap for
scientists to develop artificial blood vessels, as their publication marked the birth of vascular tissue engineering.

### 3.2 First clinical use of a TEBV

Congenital cardiac anomalies, a diverse spectrum of defects, result in significant perinatal morbidity and mortality. Untreated single ventricle anomalies are associated with 70% mortality in the first year of life. The therapy of choice is surgical reconstruction. Without surgery, survival of this cohort into adulthood is rare. Initially described in 1971, the Fontan operation separates pulmonic and systemic blood flow; subsequently decreasing the incidence of chronic hypoxia and high output cardiac failure. This suite of procedures requires a synthetic graft, commonly Polytetrafluoroethylene (PTFE, or Gore-Tex®). As mentioned above, PTFE has several limitations, including potential for infection, thrombosis, and ectopic calcification. Furthermore, PTFE does not grow, resulting in patients “outgrowing” the graft and requiring re-operation, or suffering complications related to intentional graft over-sizing.

The fabrication and seeding of biodegradable polymer scaffolds for use in humans was an outgrowth of work performed in large animal models. Precursors to the clinical study included the creation of a tissue-engineered heart valve by Zund and others. This valve construct consisted of biodegradable polyglycolic acid fibers serially seeded with fibroblasts and endothelial cells. These tissue-engineered valvular constructs were implanted in the pulmonary valve position in juvenile lambs. Functional performance of the grafts was satisfactory on ultrasound, and histological analysis revealed appropriate cellular architecture. These results were validated by a larger study with acellular valve controls, in which the seeded scaffolds demonstrated superior functionality.

The natural extension of tissue-engineered heart valves was the creation of tissue-engineered vascular conduits. These tubular constructs could be fabricated from the same scaffold materials and cellular elements, with the additional advantage of less complex biomechanical constraints.

Although both tissue-engineered heart valves and blood vessels demonstrated mechanical integrity and vascular neotissue formation
in vivo, the original seeding models relied on time-consuming expansion of endothelial cells and smooth muscle cells in vitro. This made experiments time consuming and vulnerable to culture contamination, in addition to limiting the practicality of human use as each patient would require multiple procedures. Attention was turned to other sources of seeded cells, with a focus on cells that could be procured on the day of surgical implantation of tissue-engineered grafts. Seeking an abundant cell source that did not require ex vivo tissue culture expansion, Matsumura and colleagues seeded polymer scaffolds with autologous bone marrow and implanted these constructs into the inferior vena cava of dogs. Not only did these grafts remain patent, histological analysis revealed cell populations elaborating vascular endothelial growth factor (VEGF) and expressing endothelial or smooth muscle cell markers.

The first reported use of a tissue-engineered graft in humans occurred in 1999, in a four-year-old girl with a single right ventricle and pulmonary atresia. At the age of three she had undergone pulmonary artery angio-plasty and the Fontan procedure. Subsequent angiography revealed total occlusion of the right intermediate pulmonary artery. A 2 cm segment of peripheral vein was explanted and cells expanded ex vivo. Cells were seeded onto a caprolactone–polylactic acid copolymer scaffold, reinforced with woven polyglycolic acid. The occluded pulmonary artery was successfully reconstructed with the tissue-engineered graft. No post-operative complications were reported. On follow-up angiography, the transplanted vessel was patent with no signs of aneurismal dilation.

Promising large animal study data and the first successful human application of a tissue-engineered graft provided the impetus for a larger clinical study. From May 2000 to December 2004, 25 patients (mean age 5.5 years) with single ventricle physiology were implanted with tissue-engineered grafts at Tokyo Women’s Medical University. Extra cardiac total cavopulmonary connection (EC TCPC) conduits (a connection between the vena cava and the pulmonary arteries) for surgical correction of single ventricle physiology represented an ideal hemodynamic starting point for tissue-engineered grafts in humans. In this system, high flow rates minimize thrombotic risks while relatively low pressure minimizes wall tension on the conduit. This pilot study evaluated two types of
scaffolds, PCLA-PGA \((n = 11)\) and PCLA-PLA \((n = 14)\). Autologous bone marrow \((5 \text{ mm/Kg BW})\), aspirated from the patient’s ilium under general anesthesia, was used to seed the polymer scaffold and the construct was incubated for two hours in media prior to implantation. All 25 patients survived to hospital discharge.\(^{33}\) Duplex ultrasonography, computed tomography, magnetic resonance angiography, and cineangiography were utilized for graft surveillance (Fig. 1). All grafts remained patent, and no aneurismal dilation was detected with any imaging modality.\(^{34}\) Of note,

![Fig. 1. Three-dimensional computed tomography reconstruction of *in vivo* TEBV. Tissue engineered vascular graft in a 13-year-old with single ventricle physiology. The graft connects the superior vena cava to the pulmonary arteries. Three-dimensional reconstructed computed tomography of the heart, great vessels, and tissue engineered vascular graft demonstrate a widely patent graft with no evidence of stenosis, thrombosis, or aneurismal dilation.](image-url)
six patients developed silent graft stenosis, and four underwent successful balloon angioplasty. Four patients died during follow-up (mean follow-up 5.8 years). The causes of death, however, were not related to tissue-engineered graft dysfunction; all four patients had imaging demonstrating a widely patent graft prior to death. Of the 21 surviving patients, 18 were classified as New York Heart Association (NYHA) Class I (no impairment of physical activity) and 3 were classified as NYHA Class II (mild impairment of physical activity).

Although the Tokyo Women’s study remains a landmark in tissue engineering, this data represents a single institutions experience and patients were not randomized. Additionally, although autologous bone marrow cells remain an attractive cell source for tissue-engineered graft seeding, no histological specimens exist from this clinical trial (autopsies are not usually performed in Japan, and most patients are still alive). The fate of seeded bone marrow, the optimal seeded cell number, and the role of postoperative anticoagulation remains to be determined.

### 3.3 Sheet-based tissue engineering

In the continuing search for an ideal conduit to repair damaged human vessels, L’Heureux et al. employed a new methodology termed Tissue Engineering by Self-Assembly (TESA) to produce human vessels in vitro. TESA channels the ability of cells of mesenchymal origin to secrete and assemble large amounts of ECM in various geometries. Sheet-based tissue engineering (SBTE), a variation of TESA, employs sheets of living cells and the natural ECM they synthesized to produce tubular structures and create vessel-like structures. In developing SBTE, L’Heureux et al. sought to overcome the need to use synthetic material for structural support of biological grafts as was the case for Weinberg and Bell. In this approach only cellular components were employed in the creation of their artificial blood vessel.

The first TEVG produced by SBTE contained only human skin fibroblasts cultured from dermal specimens removed during reductive breast surgery; smooth muscle cells and endothelial cells were isolated from newborn umbilical veins or adult saphaneous veins. Nonetheless, it had a burst pressure of $2594 \pm 501$ mmHg. Cell sheets were produced in
culture medium supplemented with 50 μg/ml of sodium ascorbate, peeled off from culture flasks and tubularized around inert tubular support cylinders. After a maturation period of at least eight weeks, the sheets become cohesive tubes that can be seeded with endothelial cells. The endothelial layer of the constructs expressed von Willenbrand factor immunohistochemical staining and demonstrated ac-LDL uptake; smooth muscle cells stained positively for alpha-smooth muscle actin and desmin. Fibroblasts did not stain for smooth muscle cell markers, but expressed vimentin and synthesized elastin, which organized in circular arrays. The ECM contained laminin, fibronectin, chondroitin sulfates and various collagen subtypes. Further research demonstrated that these artificial blood vessels retained concentration dependent contractile properties, responsive to various vasoconstricting (histamine, bradykinin, ATP) and vasodilating (sodium nitroprusside, SIN-1, forskolin) agents with and without inhibitory agents.\(^{36}\)

In the following years, the model was simplified to exclude smooth muscle cells and was shown to be feasible using skin fibroblasts and venous endothelium isolated from elderly patients with cardiovascular disease. Preclinical studies in immunodeficient nude rats, evaluated for up to 225 days, demonstrated patency rates of 85% without aneurism as abdominal aorta interpositional grafts. To study the in vivo development of these grafts in a more representative biomechanical environment, the tissue-engineered blood vessels were implanted in immunosuppressed cynomolgus primates. Explants at six and eight weeks demonstrated patent vessels with no signs of luminal narrowing or aneurismal formation. Alpha-actin positive smooth muscle cells and proteoglycan expression was also observed in both models.\(^{36,37}\)

In 2007, L’Heureux and McAllister reported the second clinical use of vascular tissue-engineered constructs when they described the implantation of their tissue-engineered vascular graft in three patients undergoing hemodialysis treatment (Fig. 2).\(^{18}\) These dialysis patients had a history of previously failed access grafts. After 24 patient-months of follow-up for this cohort, only one of the grafts used for dialysis access had a thrombogenic failure, secondary to low postoperative flow rate and moderate dilatation of the graft. McAllister et al. then expanded their cohort to ten patients in total, drawing from two centers, in Argentina and in Poland.\(^{38}\)
Three out of the ten implanted grafts failed within the safety phase of their study, and one patient required surgical re-intervention to maintain patency at 11 months. These results are consistent with the expected failure rates with native hemodialysis access grafts (fistula) in such high-risk patient populations. Overall, after six months, the artificial grafts implanted as hemodialysis access grafts had a 60% primary patency rate which is superior to that of the standard of care synthetic graft (ePTFE).

### 3.4 Stem cells for vascular tissue engineering

During the past decade, significant attention has been turned to the use of stem cells in tissue engineered vascular grafts. Stem cells and endothelial progenitor cells can differentiate into vascular lineages and thus have the potential to repair vascular systems. Despite their obvious potential in clinical practice, there still remain many controversies regarding how EPCs actually enhance endothelial repair and neovascularization. Additionally, because of the limited expansion ability of EPCs, expansion of sufficient EPC populations for therapeutic angiogenesis remains a significant impediment for most investigators. On the other hand, embryonic stem
cells have an extensive self-renewal activity and can be expanded without limit, thus ES cell-derived endothelial cells may be feasible as a novel cell source for therapeutic angiogenesis. Nourse and colleagues used VEGF to induce differentiation of functional endothelium from human embryonic stem cells. Continuous VEGF treatment of embryonic stem cells resulted in a four- to five-fold enrichment of CD31(+) cells but did not increase endothelial proliferation rates, suggesting a primary effect on differentiation. CD31(+) cells purified from differentiating embryoid bodies upregulated ICAM-1 and VCAM-1 in response to TNF-α, confirming their ability to function as endothelial cells. Collagen gel constructs containing the human embryonic stem cell derived endothelial cells and implanted into infarcted nude rat hearts formed dense networks of patent vessels filled with host blood cells. Thus Nourse et al. demonstrated the ability of human embryonic stem cell derived endothelial cells to facilitate neovascularization of tissue-engineered constructs.

In addition to driving ES cells into an endothelial lineage, investigators have turned to embryonic stem cell derived smooth muscle cells as a potential cell source in cardiovascular tissue engineering. NADPH oxidase (Nox4) over-expression in embryonic stem cell culture resulted in increased smooth muscle cell marker production, whereas knockdown of Nox4 induced a decrease in production. Moreover, Nox4 was demonstrated to drive smooth muscle differentiation through generation of H₂O₂. Thus Nox4 expression maintains differentiation status and functional features of stem cell-derived smooth muscle cells, highlighting its impact on vessel formation in vivo. It is clear that embryonic stem cells and endothelial progenitor cells will be important in vascular tissue engineering in the future, and endothelial cell and smooth muscle cells derived from embryonic stem cell culture will require further characterization.

Autologous, readily available, bone marrow mononuclear cells (BMC) in both human and animal models promote angiogenesis, early endothelialization, and contain multi-potent cells that can also form part of the growing neovessel. In trying to develop a TEVG, Matsumana et al., reported initial success with the use of harvested vessel wall cells after isolation and culture. They found that this was a time consuming process that required previous hospitalization for vessel harvesting and
that in half of the cases they were not able to obtain sufficient cells on the
day of surgery. As a result, this group turned to BMC as the cell source
for their constructs, removing the impediments of long-term cell culture
and contamination, and more importantly, providing sufficient cells for
the seeding of biodegradable scaffolds.\textsuperscript{34,47–49} In the first human clinical
trial with long term follow-up,\textsuperscript{50} 25 TEVG were implanted using autolo-
gous BM derived mononuclear cells with no evidence of aneurysm
formation, graft rupture, graft infection, or ectopic calcification; four
patients had graft stenosis and underwent successful percutaneous angi-
plasty. These initial findings demonstrated the feasibility and safety of
this technique and provided evidence for the use of BMC for the con-
struction of tissue engineered vascular graft. (\textit{Clinical studies described
in detail in section 3.2.})

4. Conclusion

The history of reconstructive medicine and surgery dates back centuries
as physicians and scientists have continually sought to restore function to
damaged tissue. Vascular tissue engineering began its path towards clini-
cal utility with the construction of the first blood vessel model by
Weinberg and Bell. In the ensuing years, several major breakthroughs in
biological vascular graft production have led this young field into its first
successful clinical applications. Vigorous research continues, as these
clinical trials are in their early phase; it is a matter of time before they are
attempted in the United States under FDA guidance.

In the last 30 years we have witnessed the success, and the various
pros and cons of the methodologies used to create neovessels. The first
generation of tissue-engineered arterial grafts represent a significant step
forward, but several challenges remain to be addressed. Approaches that
rely on bioreactors require significant culture time, preclude “off the
shelf” availability and place the conduit at risk for contamination. Also,
the cell source remains challenging, as adult human SMC’s have limited
number of passages \textit{in vitro}, precluding the development of a well-
developed tunica media. Finally, standardized imaging algorithms for
implanted grafts will provide a more structured analysis of graft patency,
dilation, and/or stenosis.
Undoubtedly, new approaches and techniques have yet to be developed. Modern technology in polymer fabrication or assembly may yield a more ideal scaffolding material. Advances in cell culture techniques may allow for faster production of artificial tissue. Ultimately, a deeper understanding of the signaling cascades involved in cellular interactions in neotissue development will be critical to the construction of third generation grafts. Modification of the genetic and molecular constitution of cells may lead to the development of tissue with selective maturation properties. Employing particle release technology, grafts may be constructed that would elute growth factors, cytokines or other molecular signals that may recruit host cell development onto scaffolding material, doing away with the need for cell seeding altogether.

As this technology develops, successful clinical applicability will be paramount to future implementation. The focus in the field remains on responsible development of modern tools to combat disease that affect a large portion of the population. Potential widespread use for TEBVs will be dependent on patient safety, and functionality of TEBVs that is as least equal to currently used synthetic grafts. Through landmark accomplishments and continued clinical achievements, we near the promise of artificially constructing neotissues that replicate the function of the human body.

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Stem Cells for Vascular Regeneration: An Engineering Approach

Laura E. Dickinson and Sharon Gerecht

1. Introduction

With advances in tissue engineering and the ever-rising number of vascular diseases in western countries, vascular regeneration has emerged as a major research focus. Cardiovascular disease is the number one cause of death worldwide\(^1\) and is on the rise in developing countries, with atherosclerosis alone accounting for more than half of the deaths in western countries. Current treatments for vascular disease include a regimented schedule of statins or surgical methods to physically clear or bypass diseased blood vessels. But these methods only provide temporary relief or simply delay the onset of complications. Recently, cellular therapies have emerged as a promising approach to vascular disease. Stem and progenitor cells are capable of differentiating into vascular lineages and thus have the potential to repair injured vasculature. Delivery of stem or progenitor cells could induce angiogenesis, the formation of blood vessels from the existing vasculature, and the regeneration of diseased tissues.\(^2\)
In human vascular tissue, blood vessels are composed of an internal monolayer of endothelial cells (ECs) surrounded by a thicker external shell of smooth muscle cells (SMCs). SMCs provide structural support to stabilize endothelial cells and also regulate vascular functions during vessel growth, such as modulating blood flow and maintaining vasoconstriction. During angiogenesis, remodeling of the extracellular environment surrounding blood vessels occurs to allow the migration and integration of ECs and SMCs. For successful vascular regeneration, ECs and SMCs need to integrate with each other and with the host vasculature. Thus far, one of the major obstacles in vascular regenerative medicine is deriving cell populations \textit{in vitro} that can be translated \textit{in vivo} for patient therapy. Biocompatible scaffolds can provide microenvironments conducive to cell attachment, differentiation, and integrated three-dimensional (3D) vascular formation. This chapter discusses current approaches to vascular regeneration and therapies using stem cells (SCs): it will explore various cell sources and their potential for vascular regeneration and the engineering approaches to vascular differentiation, focusing on biomaterials for the formation of 3D vascular structures.

2. Cell Sources

2.1 Embryonic stem cells

Embryonic SCs (ESCs) are derived from the inner cell mass of blastocysts of the developing embryo; are pluripotent, capable of differentiating into every somatic cell type; and have unlimited capacity for self-renewal. \textit{In vivo}, the extracellular microenvironment presents instructive biochemical cues that govern sequential cell fate decisions and differentiation. \textit{In vitro}, spontaneous differentiation of human ESCs begins with the formation of embryoid bodies (EBs). This occurs after the withdrawal of factors that maintain ESCs in their undifferentiated state. Culturing human ESCs in suspension results in cell aggregation, differentiation, and the formation of EBs, which recapitulate limited aspects of embryonic development and contain a variety of spontaneously differentiated cell types (including integrated vasculature networks) (Fig. 1A). However, since EBs contain various cell types, spontaneous
differentiation of ECs and SMCs from EBs is not efficient. To achieve a purified culture of vascular cells, desired progenitor cell types are isolated from EBs and supplemented with exogenous growth factors or specific extracellular matrix (ECM) components to further guide differentiation.
Differentiated hESCs contain a population of vascular progenitor cells with the ability to differentiate into both endothelial-like and smooth muscle (SM)-like cells. Vascular progenitor cells, isolated from EBs at day 10 by the expression of the specific endothelial/hematopoietic marker CD34, are selectively induced to differentiate into either endothelial or SM-like cells when cultured in supplemental medium containing vascular endothelial growth factor (VEGF) or platelet-derived growth factor (PDGF), respectively (Fig. 1B). VEGF is a pro-angiogenic factor known to stimulate angiogenesis both \textit{in vitro} and \textit{in vivo}, and induce the endothelial and hematopoietic differentiation of hESCs. ECs derived from EBs form vascular structures both \textit{in vitro} and \textit{in vivo}. When ECs and SM-like cells embedded in Matrigel scaffolds were implanted in severe combined immunodeficient (SCID) mice, Ferreira \textit{et al.} reported the formation of microvasculatures that functionally integrated with the hosts (Fig. 1B). As both endothelial and SMCs are integral in blood vessel formation, implanting both hESC-derived cell types may provide more effective vascular regenerative therapies.

Spontaneous and induced differentiation of ESCs can occur through 3-dimension EB formations, as well as in 2-dimension adherent cultures. Endothelial progenitor cells have been isolated from EBs and cultured two-dimensionally for final differentiation into vascular cell lineages. Two-dimensional differentiation protocols incorporate cell feeder layers or ECM components that provide specific secreted biochemical molecules or proteins known to promote SC attachment and enhance differentiation. Various ECM components, such as collagen or fibronectin, have been implicated for their role in guiding differentiation. These macromolecules provide cell adhesion sites and influence cell fate through integrin-mediated signaling events. Collagens are the most abundant ECM component. Specifically, collagen IV is implicated in mesodermal differentiation. Undifferentiated ESCs cultured on collagen IV substrates present an efficient mesodermal differentiation model. Enhanced differentiation to ECs or SMCs is observed with supplementation of VEGF or PDGF, respectively.

Co-culturing pluripotent SCs with terminally differentiated cells provides an interactive environment that allows the exchange of secreted cytokines and regulatory signals necessary for cell fate decisions. Culturing
differentiating SCs with specific stromal cell lines to promote vascular differentiation has been extensively studied. Human ESCs have been differentiated in vitro into hematopoietic precursors on 2D mouse bone marrow (BM) cells and endothelial feeder cells. Co-culturing hESCs with BM or yolk sac ECs can lead to differentiation into early hematopoietic precursors with the potential to differentiate into mature ECs. ECs and SMCs derived from hESCs already demonstrate regenerative potential in facilitating neovascularization and improving blood flow in vascular injury models, and therefore are a promising source for cell-based regenerative therapies.

The successful differentiation of SCs to ECs or SMCs is characterized by cellular morphology, function, and the presence of specific cellular markers and proteins. ECs display a cobblestone morphology and express specific endothelial markers, such as vascular endothelial cadherin (VE-cad), platelet EC adhesion molecule-1 (PECAM1), CD34, vascular endothelial growth factor receptor 2 (VEGFR2), and von Willebrand factor (VWF). Functional ECs also have the capacity to form capillary-like structures in vitro; to express endothelial nitric oxide synthase (eNOS), a protein that generates nitrous oxide in blood vessels and regulates vascular function; and to uptake vascular EC labeling reagent Dil-Ac-LDL. SMCs, on the other hand, have a spindle-shaped morphology and exhibit the functional ability to contract/relax in response to pharmacological agents. In addition, SMCs express specific markers restricted to contractile MCs; α-SM actin, SM myosin heavy chain, calponin, caldesmon, SM22, and smoothelin.

2.2 Mesenchymal stem cells

Mesenchymal SCs (MSCs) are multipotent SCs derived mainly from BM or adipose tissue. Unlike hESCs, MSCs are easy to isolate and expand, and differentiate into cell types of mesodermal lineages, including vascular cells. MSCs have demonstrated the ability to regenerate vessel integrity at sites of vascular injury and to enhance angiogenesis through the release of pro-angiogenic factors. Supplemental VEGF and basic fibroblast growth factor (bFGF), known to mediate proliferation and migration of MSCs, can successfully induce differentiation of BM
MSCs into vascular endothelium-like cells.\textsuperscript{18} MSCs have been shown to differentiate \textit{in vitro} into SMCs and to form blood vessel walls similar to native vessels,\textsuperscript{19} making them another potential cell source for vascular regeneration applications and therapies.

\subsection*{2.3 Endothelial progenitor cells}

Progenitor cells are another viable cell source for vascular regeneration therapies. Endothelial progenitor cells (EPCs), first isolated from human peripheral blood in 1997,\textsuperscript{20} are capable of differentiating into mature ECs in response to such stimuli as growth factors, cytokines, and mechanical shear stresses.\textsuperscript{21,22} EPCs are capable of mobilizing from BM to ischemic tissue to facilitate neovascularization.\textsuperscript{20} This process is initiated by hypoxia, which induces the production and secretion of pro-angiogenic factors, such as VEGF, and recruits EPCs to sites of injury. Isolation of EPCs from additional sources (beside the peripheral circulation and BM) has been demonstrated, including umbilical cord blood, liver tissue, or vascular walls themselves.\textsuperscript{23–26} Unlike pluripotent SCs, progenitor stem cells differentiate into a predetermined cell type and have a finite capacity for self-renewal. However, EPCs have the benefit of providing patient-specific therapies since these cells can be isolated from individual patients.

\subsection*{2.4 Induced pluripotent stem cells (IPSCs)}

Human somatic cells, such as fibroblasts, transfected with stem-cell associated genes can be reprogrammed (induced) to pluripotent SCs (IPSCs) that exhibit the essential characteristics of hESCs.\textsuperscript{27,28} IPSCs maintain the developmental potential to differentiate into cell types from all three germ layers and could allow the generation of patient-specific pluripotent cells for regenerative medicine.\textsuperscript{27} Recently, human IPSCs have been differentiated into both ECs and mural cells by culturing on OP9 murine BM cells, the same method used to differentiate hESCs.\textsuperscript{29} The ECs and mural cells differentiated from IPSCs displayed nearly identical properties to those from hESCs, indicating the potential of IPSCs for vascular regeneration therapies.
3. Engineering Vascular Differentiation

Many different approaches have been employed to differentiate organized vascular structures from embryonic and adult SCs, both in vitro and in vivo. As discussed above, instructive biochemical cues, such as growth factors or ECM components, provide culture environments that guide and regulate the efficient differentiation of SCs to vascular cell types. However, engineering microenvironments that incorporate the biomechanical or biophysical cues observed during angiogenesis also direct vascular differentiation. Herein we briefly discuss how mechanical stimulation or surface topography is utilized to induce SC differentiation to endothelial and smooth muscle cell phenotypes.

3.1 Mechanical stimulation

In addition to growth factors, mechanical forces are also known to regulate cellular functions and to induce lineage-specific differentiation of SCs in vitro. Mechanical stimuli that mimic the physiological environments of blood vessels have been shown to accelerate the endothelial maturation of EPCs and to induce differentiation of murine ESCs. Reproducing the shear stress that cells experience during blood flow in arteries stimulates cellular differentiation. By exposing ESC cultures to a laminar flow, cells proliferate and differentiate into vascular ECs, whereas cyclical strain induces vascular SMC differentiation. Under a pulsatile flow loading system that simulates both hydrodynamic shear stress and circumferential strain, as would be experienced in human veins, ESCs differentiated into vascular wall cells, including both endothelial-like and SM-like cells simultaneously. Another recently engineered system subjected hMSCs grown on tubular silicone substrates to shear flow, radial flow, and pulsatile pressure to induce the expression of ECs and SMCs.

3.2 Surface topography

The vascular microenvironment is a topographically complex milieu, providing the structural platform necessary for tube morphogenesis. Many of the cellular interactions within this environment occur in the micron
and sub-micron regime. In fact, synthetic micro- and nanoscale substrates have been shown to influence the cellular functions of ECs and SMCs via contact guidance, and induce their organization into vascular structures.\textsuperscript{34,35} Human EPCs, when cultured on submicron line grating substrates, align in the direction of the features and are capable of forming supercellular band structures compared to hEPCs grown on flat substrates.\textsuperscript{34} The addition of Matrigel induced hEPCs to form extensive capillary networks with longer average tube lengths than hEPCs merely grown on flat substrates (Fig. 2A). This indicates that hEPCs, as well as ECs and SMCs, can potentially be stimulated by topographical cues to form vascular structures \textit{in vitro}.

\section*{4. Three-Dimensional Space}

Reconstructing 3D environments that mimic biochemical and biophysical cues \textit{in vivo} for vascular differentiation and functional regeneration is a developing field of research. In hESC biology, the surrounding 3D microenvironment influences cellular function and differentiation through interactions with the ECM components and neighboring cells. The ECM is highly involved in vascular development and regeneration by providing the scaffold necessary to stabilize the organization of ECs to functional endothelium within its integrated fiber and protein networks,\textsuperscript{36} while also presenting instructive biochemical cues to regulate and support cell proliferation, migration and differentiation.\textsuperscript{37} The ECM provides structural support for the vascular endothelium by acting as a scaffold to stabilize the organization of ECs into blood vessels, as well as support cell proliferation, migration, and survival.\textsuperscript{38} Therefore, \textit{ex vivo} vascular engineering generally involves the use of scaffolds which are designed to provide 3D structural and logistical templates for tissue development, to control the cellular microenvironment, and to provide the necessary molecular and physical regulatory signals. Thus, the development of a 3D scaffold compatible with vascular stem and progenitor cells will provide an experimental approach to direct vascular differentiation and assembly.
4.1 Biomaterials for vascular differentiation

Biomaterial scaffolds provide a 3D support to mimic the ECM and promote cell attachment and growth and are of synthetically biodegradable materials, natural materials, or a composite of both. Indeed, biomaterials have been fabricated into porous, fibrous, or hydrogel scaffolds; each with distinct properties, as summarized in Table 1. A fibrous scaffold mimics the
<table>
<thead>
<tr>
<th>Polymer</th>
<th>Natural/Synthetic</th>
<th>Reason for Use</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>Natural polysaccharide</td>
<td>Gels under gentle conditions</td>
<td>Low toxicity</td>
<td>Loss of mechanical stiffness over time when ionically crosslinked</td>
<td>Allows efficient EB formation; induces vasculogenesis in EBs$^{39}$</td>
</tr>
<tr>
<td>Dextran</td>
<td>Natural polysaccharide</td>
<td>Functional groups make it amenable to modifications</td>
<td>Biodegradable; biocompatible</td>
<td>Not enzymatically degradable in vivo; slow tissue integration</td>
<td>Enhances vascular differentiation of hESCs and maturation of EPCs</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>Natural polysaccharide</td>
<td>ECM component, ubiquitous during development; regulates angiogenesis</td>
<td>Biocompatible, biodegradable</td>
<td>Poor mechanical properties and cell attachment</td>
<td>Supports hESC propagation; stimulates EC proliferation</td>
</tr>
<tr>
<td>Fibrin</td>
<td>Natural protein</td>
<td>Involved in blood clotting, in situ capillary formation</td>
<td>Biocompatible; produced from patients (no potential inflammatory responses)</td>
<td>Poor mechanical properties</td>
<td>Supports BM progenitor cell differentiation and vessel formation</td>
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Table 1. (Continued)

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<tr>
<th>Polymer</th>
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<th>Reason for Use</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (l-lactic acid) (PLLA)</td>
<td>Synthetic</td>
<td>Provides mechanical stiffness</td>
<td>Supports 3D structures</td>
<td></td>
<td>Promotes 3D vascularization in vivo</td>
</tr>
<tr>
<td>Poly(lactic-co-[glycolic acid])</td>
<td>Synthetic</td>
<td>Facilitates cellular and tissue ingrowth</td>
<td>Quickly degrades into easily metabolized products</td>
<td></td>
<td>Promotes in vivo ingrowth of vascular tissue, cell adhesion, and proliferation</td>
</tr>
<tr>
<td>Poly (glycolic-co-sebacate) acrylate (PGSA)</td>
<td>Synthetic</td>
<td>Elastomeric material that mimics ECM</td>
<td>Biocompatible</td>
<td></td>
<td>Promotes tissue ingrowth, integrated vascularization in vivo, hESC encapsulation, proliferation</td>
</tr>
<tr>
<td>Polycapro-lactone</td>
<td>Synthetic</td>
<td>Long-term degradation supports in vivo applications</td>
<td>Biocompatible; biodegradable into fragments eliminated by macrophages; high elasticity(^4)</td>
<td></td>
<td>Supports EC adhesion, proliferation, and spreading</td>
</tr>
</tbody>
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3D — three-dimensional, BM — bone marrow, EB — embryoid body, EC — endothelial cell, ECM — extracellular matrix, EPC — endothelial progenitor cell, hESC — human embryonic stem cell.
fiber network of the native ECM (composed predominantly of collagen and elastin), while porous scaffolds are produced with macroscopic voids that enable cellular interaction. Hydrogels are water-swollen, crosslinked polymers fabricated from synthetic or natural biomaterials that provide structural support and can direct differentiation of hESCs to form vascular networks \textit{in vitro}. Because their capacity to absorb and retain large amounts of water mimics the structural properties of the ECM, hydrogels have been investigated for their potential as drug delivery devices, tissue engineering scaffolds, and cell culture substrates. Furthermore, hydrogels can be engineered specifically for vascular regeneration applications by optimizing controllable mechanical, degradation, and structural properties via compositional modifications, crosslinking ratios, etc.

Ideal scaffolds have been identified as having the following properties:

1. Porous and permeable to allow cell growth, migration, and interaction, as well as the transport of nutrients,
2. Biodegradable to match \textit{in vivo}/\textit{in vitro} tissue growth with nontoxic, easily eliminated by products,
3. Biocompatible and with a surface chemistry suitable for promoting cell attachment, proliferation, and differentiation,
4. Mechanically favorable to support cellular organization, and
5. Shapeable, to conform to different geometries and sizes.

These properties are controllable in scaffold design to generate a construct that can support and guide the regeneration of vascular tissue either \textit{in vitro} or \textit{in vivo}.

4.1.1 \textit{Natural biomaterials}

Biomaterials derived from ECM components or plants are naturally bioactive, biocompatible, and biodegradable. Alginate, one such naturally derived biomaterial, is a polysaccharide with homopolymeric blocks of 1,4-linked \( \beta \)-D-mannuronic and \( \alpha \)-L-gluuronic residues and is isolated from the cell walls of brown seaweed. Alginate polymers can be ionically crosslinked to form a hydrogel by addition of divalent cations, calcium,
and barium, and have been extensively studied as 3D scaffolds compatible with many cell types for culture and encapsulation. In fact, 3D porous alginate scaffolds promote efficient EB formation with a high degree of cell proliferation and differentiation when used to culture undifferentiated hESCs. In fact, EB formation is predominantly restricted to scaffold pores, resulting in highly vascularized EBs containing microvascular networks and tube-like structures. This indicates that the physical confinement exerted by the porous alginate scaffold is sufficient to induce vasculogenesis in differentiating EBs. However, alginate is not enzymatically degradable in mammals and therefore poses potential barriers for tissue integration with host vasculature.

Another biomaterial, dextran, is a natural, branched polysaccharide synthesized from sucrose using lactic acid bacteria, such as Streptococcus mutans, and is composed of α-1,6-linked D-glucopyranose residues. Pendant functional groups, such as –OH, make dextran amenable to chemical modifications for greater flexibility in the formulation of dextran-based hydrogels. Modifying dextran-based hydrogels with cell adhesive ligands, such laminin- or fibronectin-derived Arg-Gly-Asp (RGD) peptides, increases the potential for using dextran hydrogels for tissue engineering applications by enhancing cell adhesion and survival. Recently, bioactive dextran-based hydrogels were shown to enhance the vascular differentiation of hESCs. Ferreira et al. showed that encapsulated, undifferentiated hESC aggregates differentiated and formed EBs in RGD-modified dextran-based hydrogels with microencapsulated VEGF. At day 10, encapsulated EBs showed increased expression and localization of the endothelial markers CD34 and PECAM1, along with the formation of a primitive vascular network. When the cells were removed from the dextran hydrogel encapsulation and cultured in vascular differentiation media, specific proliferation occurred along the vascular lineage. This demonstrates the potential of functionalized dextran hydrogels to derive large quantities of ECs for vascular regeneration from hESCs. Dextran-based hydrogels can also function as 3D scaffold materials for potential in vivo transplantation. Bifunctional dextran, modified with methacrylate and aldehyde with incorporated gelatin, mimics the complex composition and topography of the ECM. These novel dextran-modified hydrogels promote EC adhesion and support the spreading and proliferation of SMCs.
One of the chief components of the ECM is the glycosaminoglycan hyaluronic acid (HA), a non-sulfated linear polysaccharide consisting of glucuronic acid and N-acetylglucosamine residues. HA is involved in many hESC cellular processes, including adhesion, morphogenesis, proliferation, and motility in vivo. The regulation of these cellular events is attributed to the interaction of HA with the cell surface receptors CD44 and receptor for HA Mediated Mobility (RHAMM), which transduce intracellular signals. CD44 is known to mediate HA-induced cell proliferation and survival pathways, while RHAMM is involved in HA-induced cell motility, signaling, and differentiation via extra- and intracellular pathways. RHAMM also has been implicated in the maintenance of hESC pluripotency, viability, and cell cycle control. Undifferentiated hESCs have been found to express high levels of HA, CD44, and RHAMM in culture. Similarly, the developing embryo is surrounded by a high concentration of HA during early embryogenesis, which decreases with the onset of differentiation. In fact, the inner cell mass cells of the developing embryo are embedded in HA-rich 3D matrices that regulate their self-renewal and differentiation. In addition to a role in embryonic development, HA regulates angiogenesis by stimulating EC proliferation, migration, and sprouting. HA hydrogels were recently investigated to determine their effect on hESCs in vitro. They were discovered to create a microenvironment conducive to the propagation of undifferentiated hESCs. When encapsulated in 3D hydrogels composed entirely of HA, colonies of hESCs maintained their undifferentiated state of self-renewal and preserved their normal genetic integrity. EB formation from hESC-HA released cells, indicates that hESCs cultured in HA hydrogels retain their full differentiation potential, and can switch to vascular differentiation with the introduction of angiogenic factors. The addition of VEGF to the differentiation medium in HA gels induced cell sprouting and elongation within 48 hours. The presence of specific endothelial and SM markers, CD34 and α-SM actin, confirmed that these cells are indeed of vascular lineages (Fig. 2B). These HA hydrogels could be used as a method to enhance the propagation of hESCs being further differentiated into vascular/endothelial lineages for regeneration applications. Already, HA-based scaffolds have proven to be a promising biomaterial for vascular regeneration applications. HA-based scaffolds have been instrumental in developing tissue
engineered vasculatures *in vitro* and orchestrating the vascular remodeling events necessary for small artery reconstruction *in vivo*.51

Fibrin is the major structural, fibrous protein involved in blood clotting and in remodeling the ECM during wound healing. Capillary growth *in situ* frequently occurs in a fibrin-rich extracellular environment. When adult BM progenitor cells are cultured in 3D fibrin matrices *in vitro*, vascular structures develop that express endothelial markers (VWF, CD34, VEGFR-2) and are surrounded by mural cells expressing α-SM actin.52 Not only do fibrin scaffolds support vascular differentiation and regeneration *in vitro*, but implanted fibrin gels seeded with BM-derived ECs and SM progenitor cells form vessels that integrate with native vasculature.53

4.1.2 Synthetic materials

Alternatively, synthetic materials have also been investigated for vascular regeneration applications. Advantages of using synthetic materials include the manipulation of tunable physical parameters, such as mechanical integrity. Many polyester-based synthetic polymers are being investigated because of their mechanical strength, biocompatibility, and degradation into easily metabolized products, such as glycolic acids, which are eliminated as carbon dioxide and water.54

Poly(lactic acid), which is produced through the polymerization of lactic acid, has a high mechanical strength in the form of poly(L-lactic acid) (PLLA). Synthetic materials can be modified with ligands to enhance the cellular adhesion of anchorage-dependent cells, like EPCs. PLLA scaffolds grafted with RGD peptides support the *in vitro* growth and endothelial functions of EPCs, as well as promote vascular regeneration in murine wound models *in vivo*.55 Fibers of PLLA interwoven with polyglycolic acid (PGA) form a biodegradable scaffold conducive to engineering microvessels *in vitro*.56 EPC-derived ECs co-seeded with SMCs on this bipolymeric (PGA-PLLA) scaffold spontaneously formed capillary and microvascular-like structures. The porous architecture of these scaffolds provide a favorable environment for microvessel formation, and ECs cultured with SMCs formed $76 \pm 35$ vessels/mm$^2$.56

PGA-based copolymers, such as poly(lactic-co-glycolic acid) (PLGA), are also used for vascular regeneration applications. PGA is copolymerized
with PLA, and can create various forms of PLGA, depending on the glycolide to lactide ratio. Shaping biomaterials into geometries that mimic vasculature is another technique to promote vessel formation. Cylindrically processed PLGA scaffolds promote the in vivo ingrowth of vascular tissue and facilitate the adhesion and proliferation of SMCs, with seeded ECs forming lumens, demonstrating the benefits of PLGA as a scaffold material for vascular regeneration studies. Immobilization or coating of bioactive molecules can further enhance scaffolds for vascular regeneration. Incorporating the angiogenic factor VEGF into PLGA scaffolds for its controlled release induces the differentiation of human MSCs into ECs. Composite scaffolds have been generated to optimize the properties of different materials and have been shown to effectively enhance vascular formation. Biodegradable scaffolds consisting of a 50/50 blend of PLLA and PLGA have been fabricated to direct differentiation and to organize hESCs into tissue-like structures. PLLA provides the mechanical stiffness to support 3D structures, and PLGA degrades quickly, facilitating cellular ingrowth. PLLA/PLGA scaffolds coated with Matrigel or fibronectin promote the cell attachment and survival of early differentiating hESCs (EBs). When supplemented with growth factors, such as retinoic acid, transforming growth factor B (TGF-B), activin A, or insulin-growth factor (IGF), 3D vascular network formation — in addition to liver, cartilage, and neural tissues — is observed. When transplanted into SCID mice, the constructs integrated with the host vasculature, indicating the potential of polymer scaffolds for directed in vitro 3D differentiation and in vivo transplantation. In later studies, hESC-derived ECs cultured with mouse myoblasts and embryonic fibroblasts formed large vessels within the PLLA/PLGA composite constructs. The embryonic fibroblasts promoted stabilization of the vessel structures by differentiating into SM-like cells (confirmed by SM actin immunofluorescence staining) and co-localizing around the hESC-derived ECs. Implantation into SCID mice improved the vascularization and survival of the skeletal muscle constructs, as confirmed by integration with the host vasculature. In vitro vascularization of constructs with successful in vivo integration is the major obstacle in tissue engineering, and many studies have attempted to solve this problem.

Synthetic elastomeric materials such as poly (glycerol sebacate) (PGSA), may better mimic the ECM of native blood vessels and are being investigated for potential use in vascular regeneration. Photocurable PGSA forms a
uniform, porous, and flexible scaffold suitable for cell encapsulation and hESC culture. Undifferentiated hESCs seeded in PGSA scaffolds proliferated and formed EBs within one week. Biocompatibility, tissue ingrowth, and integration with the host vasculature were observed after transplantation, indicating the potential of PGSA as a promising material in vascular regeneration.61

Other synthetic polymers that have been investigated for vascular regeneration include poly(ethylene glycol) (PEG) and polycaprolactone. PEG is a polymer of ethylene oxide, and PEG-based hydrogels have been used to encapsulate SCs and direct their differentiation.62 PEG is an inherently hydrophilic material, but modification with adhesive peptides enhances cell adhesion and interaction with PEG-based scaffolds.63 A recently developed synthetic PEG-based hydrogel incorporates crosslinked matrix metalloproteinase (MMP)-sensitive peptides, matrix-bound RGD peptides, and the bioactive peptide thymosin β4 (Tβ4) to more accurately mimic the natural collagenous ECM of vascular networks in vivo.64 Incorporating MMP-sensitive peptides produced a hydrogel matrix capable of cell-mediated proteolytic degradation and remodeling,65 essential for vasculogenesis, while incorporating Tβ4 in the PEG-based hydrogel allowed EC adhesion, migration, and vascular-like organization in vitro.

Polycaprolactone, another biodegradable polyester, has already been approved by the US Food and Drug Administration for vascular regeneration. Using polycaprolactone has the major advantage of slowly degrading in vivo into fragments that are easily eliminated by macrophages.54 Tubular nanofiber polycaprolactone scaffolds support EC adhesion, proliferation, and spreading, proving to be a viable option for vascular grafts using SC derived ECs.66 A scaffold composed of polycaprolactone-polylactic acid copolymer, reinforced with polyglycolic acid and seeded with vein cells, was successfully transplanted into a four-year-old patient and successfully reconstructed her pulmonary artery.67

4.1.3 Shaped scaffolds

For vascular regeneration and remodeling, scaffolds of a tubular structure are desirable to direct vascular assembly and structure formation. Tubular scaffolds have been engineered using electrospinning techniques, which produce ultrathin synthetic fibers of a mesh, imitating fibrils of ECM.68
Soletti et al. recently developed and tested a bilayer poly(ester-urethane)urea-based scaffold obtained by combining electrospinning and a phase separation technique. The bilayer scaffold presented two concentric layers, with a highly cellularized, porous internal layer mimicking the tunica media and a fibrous, external electrospun layer as the adventitial layer. Studies found that the mechanical properties of the scaffold were physiologically consistent and comparable to native vessels, and muscle-derived SCs cultured on these scaffolds under dynamic conditions maintained a high cell density.68 Recently, functional vessel walls have been engineered in vitro by optimizing these factors. Scaffolds coated with the ECM matrix protein fibronectin and subjected to cyclic strain induced human MSCs to differentiate into SMCs and form small-diameter human vessel walls.19

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References


1. Clinical Burden of Wound Healing

Wound healing and the approach to acute and chronic wounds are clinical concerns that are not always adequately addressed by current therapeutic interventions. The ability to heal wounds can complicated by multiple factors, including — but not limited to — vascular disease, diabetes, chronic disease, smoking, infection, radiation therapy, and immunocompromise. Scars forming after injury are problematic in that they can be disfiguring and limit the functional integrity of the dermis.

Diabetes has become both a national and global health concern, as the CDC estimates approximately 23.6 million people or 7.8% of the United States population currently have the disease, diagnosed or undiagnosed. In 2006, diabetes was the seventh leading cause of death in the United States, and in 2004, approximately 71,000 non-traumatic lower-limb amputations were performed in diabetic patients. In 2003, the number of diabetic patients worldwide was estimated at 197 million, and this number...
is projected to increase to 366 million by 2030 due to increased longevity.\(^2\) Limb ulcerations, infections, Charcot neuroarthropathy, and amputations are estimated at billions of dollars per year in healthcare costs. Non-healing lower extremity diabetic ulcers account for approximately 25\%–50\% of all hospital admissions in the diabetic population and are responsible for the majority of resultant amputations.\(^3\)

Current therapeutic interventions focus on controlling factors implicated in poor wound healing. To limit infection, patients are given systemic antibiotics, and chronic diseases, such as diabetes, are medically controlled. Devitalized tissue can provide nutrition for the wound’s bacterial load and impair defenses. Under circumstances where there is an abundance of debris, debridement can be used to clean the wound bed and allows for reduction of pressure and evaluation of tracking and tunneling. Debridement can be accomplished through a variety of methods: surgical, biological, enzymatic, autolytic, or mechanical. Debridement must always precede the application of topical agents and dressings.\(^4\)

Various dressings and topical agents are used to prepare the wound bed. Tissue moisture balance is important to maintain in order to promote granulation tissue and autolytic processes and prevent either dessication or the accumulation of excess fluid. Infection, which can be local, ascending, or systemic, must be identified and treated with topical agents and systemic antibiotics. Dressings can be classified as passive, providing only occlusive function, or active and interactive, modifying the wound environment physiology.\(^5\)

Despite these current therapeutic approaches in clinical practice, wounds continue to underheal, overheal, and scar. Growth factor therapy has shown great promise in treating wounds. For example, becaplermin gel, a DNA recombinant platelet-derived growth factor, has been shown to stimulate the mechanisms of wound healing.\(^6,7\) Other growth factors currently under investigation include VEGF, fibroblast growth factor, and keratinocyte growth factor.\(^4\) However, more studies are needed in addressing the unmet need for effective wound repair and regeneration. As such, stem cell-based therapy is promising as a novel way to approach wounds and shift the balance from scarring to regeneration of tissue.
2. Physiology of Wound Healing

When tissue is injured, organisms naturally protect themselves against water loss and the invasion of microorganisms by initiating wound healing. There are three main clinical outcomes when human adult tissue encounters injury. First, the tissues can heal slowly and inadequately. Second, the tissues can overheal and deposit an overabundance of fibrotic wound matrix, as is seen with hypertrophic scars and keloids. Finally, there is the overriding issue that all injured tissue heals with a scar.

In some eukaryotes, tissue can regenerate in response to injury so that it approaches its original phenotype and function. Human skin has this capacity during prenatal development, where it has been observed to heal without scar. However, through an unknown mechanism, this capacity is lost in adult life. Instead, most tissues respond to a wound by creating a patch of cells (predominantly fibroblasts), deposit disorganized extracellular matrix (predominantly collagen), finally resulting in scar formation.

Wound healing occurs classically in three overlapping phases — inflammation, tissue formation, and tissue remodeling (Fig. 1). With tissue injury, the vascular network is disrupted, and platelets are attracted to the wound to initiate hemostasis by formation of a platelet plug. At the same time, platelets secrete various mediators of wound healing, including platelet-derived growth factor. These mediators activate macrophages and fibroblasts to migrate to the wound.

Neutrophils are the first responders during the inflammatory stage. These leukocytes migrate from local blood vessels into the wound, cleansing the area. Macrophages then engulf debris. The proliferation process then ensues, during which fibroblasts, macrophages, and vascular tissues enter the wound to begin formation of granulation tissue. Fibroblasts and myofibroblasts lay down connective tissue rich in collagen, and the wound contracts through the action of myofibroblasts and matrix remodeling.

Re-epithelialization begins once granulation tissue is created, during which keratinocytes migrate over the granulation tissue to create a new layer of epidermis. In a final remodeling stage, the granulation tissue matures, during which time the synthesis and turnover of structural
Fig. 1. Classic stages of wound repair. Inflammation (A), new tissue formation (B), and remodeling (C). Originally published by Gurtner et al.\textsuperscript{67}
proteins such as collagen continues over a period of six to 24 months. At the end of this process, the wound is healed with a scar, which approaches approximately 70% of the tensile strength of the original tissue.\textsuperscript{10}

Overhealing of tissues has been described for over 200 years. Keloids were first described by Alibert in 1806 as tumorous growths or “chancreoids,” a name he later amended to “cheloid” as he developed better understanding of these growths.\textsuperscript{11} Keloids and hypertrophic scars are benign dermal growths of fibrous tissue that occur in those with a predisposition, usually in those with dark pigmented skin. These lesions often cause disfigurement and symptoms such as pain, burning, and itching. Keloids and hypertrophic scars can result from any mechanism of injury, such as trauma, inflammation, surgery, or burns, although they have also been described as occurring spontaneously.\textsuperscript{12} Histology shows over-deposition of collagen and glycoprotein in the scar tissue. Therefore, keloids and hypertrophic scars represent a derailment of the mechanism of protective wound-healing, and difficulty in delineating at which point derailment occurs has made these lesions notoriously challenging to treat.\textsuperscript{13}

In mammals, scars result from rapid deposition of fibrotic tissue, which is likely a protective mechanism developed to prevent infection and mechanical deformation. Though this rapid deposition clearly has its benefits, scar formation can be detrimental, as it prevents the regenerative process that is seen in other organisms. In order to shift the balance from scar formation to regeneration, it is thought that therapies must focus on limiting the rapid fibrotic response of tissues to injury to allow multipotent cells, such as stem or progenitor cells, promote regeneration.\textsuperscript{14} The early inflammatory phase has been identified as a target to slow the deposition of fibrotic tissue and formation of scar.

After hemostasis is achieved with clot formation, stages of wound healing begin with the inflammatory cascade and proceed to proliferation, matrix remodeling, and scar formation. The early inflammatory phase that sets in within minutes of injury largely determines areas where scar will form, as it creates signals for cytokines and growth factors dictating movement and organization of cells. As a result of stress, during the early inflammatory phase, neutrophils infiltrate the injured area, elaborating neutrophil-specific enzymes such as metalloproteinases and collagenases. These substances, along with macrophages that invade the area, break
down large amounts of tissue with free radicals and leave the injured area devoid of matrix. Ultimately, through migration and proliferation of fibroblasts, collagen production, collagen deposition, and angiogenesis, this area is filled with scar tissue.\textsuperscript{9}

As a result of the inflammatory cascade’s role in scar formation, the necessity of inflammation to the proper healing of wounds has been debated. Recent knockout and knockdown studies suggest that regulation and limitation of inflammatory infiltrates to the wound bed may, in fact, enhance wound healing. To illustrate this point, in 2003, Martin \textit{et al.} reported data on PU.1 knockout mice devoid of macrophages and functioning neutrophils. These mice were shown to heal at a similar rate to wild type mice but without the formation of scar. The authors found that the cytokine and growth factor profiles in these wounds differed from those in the wild type. As a result, cell death was reduced and scar formation was mitigated.\textsuperscript{15}

Additional studies have been performed on other knockout mice, confirming the suspicion that inflammatory cells may not be essential to wound healing. Szpaderska \textit{et al.} studied mice with induced thrombocytopenia, showing that, as long as hemostasis is achieved, platelets are not necessary for the normal healing process.\textsuperscript{16} Egozi \textit{et al.} experimented with mast cell deficient mice. The data from this group shows that the absence of mast cells did not affect overall wound healing compared to the wild type. The authors noted, however, that these mice produced decreased numbers of neutrophils early in the inflammatory phase, suggesting that the few mast cells present at the beginning of wound healing are involved in creating early activating signals.\textsuperscript{17} Athymic mice and antisense gene knockdown experiments have further confirmed the inflammatory response as an appropriate target for scar treatment and prevention.\textsuperscript{18–20}

Proliferation, matrix remodeling, and scar formation involve complex interaction between cells, cytokines, growth factors, and extracellular matrix components. This process can last up to two years after injury. Many targets in the process of inflammation and collagen synthesis and lysis have been identified to modify wound healing and promote regeneration, including tumor necrosis factor (TNF-\textit{α}), platelet-derived growth factor (PDGF), transforming growth factor (TGF-\textit{β}), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF).\textsuperscript{21} These targets
are under continual investigation for improving the regenerative capacity of tissue.

3. Stem Cells and Wound Repair

3.1 Overview of stem cell biology

During cellular development, two distinct lineages emerge as a morula becomes a blastocyst: the trophoectoderm and the inner cell mass. Embryonic stem cells, undifferentiated cells with renewal capacity, are produced from the inner cell mass and are able to divide multiple times while still remaining in an undifferentiated state. These cells are pluripotent and can, accordingly, differentiate to any of the three primary germ layers — endoderm, mesoderm, or ectoderm (Fig. 2).

In culture, embryonic stem cells can be maintained as undifferentiated cells or induced to differentiate into specific cell lineages. Development of embryonic stem cell research has allowed for genetic modification, as

![Fig. 2. Stem cell generation and differentiation. Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst. These ES cells can be induced to differentiate into cells of all three primary germ layers: endoderm, mesoderm, and ectoderm.](image)
well as the study of signals and differentiation steps involved in tissue development. This research has allowed for the creation of knockout mice and the study of specific genes involved in biological processes. However, the use of embryonic stem cells remains controversial, as ethical concerns arise regarding human cloning and the industrial production of embryos to obtain cells for study, as well as the potential of these cells to become neoplastic. As a result, attention has somewhat been redirected to the adult stem cell population as alternative source of cells with potential to remodel diverse tissues and organs.\textsuperscript{23}

Adult stem cells retain many of the same characteristics of embryonic stem cells in that they have self-renewal capabilities, long life, high potential for proliferation, and multipotency. However, these cells are typically more restricted in their ability to differentiate, and they often are lineage specific.\textsuperscript{24}

Adult stem cells are necessary for regeneration of tissue. In many tissues, stem cells are in a quiescent state until stimulated to revert back to the cell cycle to divide. Once stimulated by extracellular cues, these cells produce undifferentiated progenitor cells without self-renewal capabilities. These cells then differentiate into effector cells. The ability for stem cells to remain dormant for long periods of time allows these cells to largely avoid the risks of DNA replication and mitosis, such as mutation and exposure to harmful metabolites.\textsuperscript{25}

Adult stem cells can be found in the bone marrow or in the tissues themselves. Tissue stem cells have been found to have a large role in maintaining homeostasis in areas such as the skin’s epidermis, which displays a fast rate of cell turnover and high regenerative potential. When cell division and regeneration go awry in such tissues, malignancy can result. Tissue stem cells exist in functional niches, which are tissue microenvironments that shelter stem cells from stimuli for replication and apoptosis, maintaining a balance between activity and quiescence.\textsuperscript{26}

### 3.2 Epidermal stem cell biology

The cutaneous epithelium is constituted by epidermis, dermis, hair follicles, sebaceous glands, and sweat glands. The outer epidermis is separated from the inner dermis by a basement membrane. Human
interfollicular epidermis is a constantly renewed, multilayered stratified squamous epithelium made up of keratinocytes. The proliferation of keratinocytes in the basal layer of the interfollicular epidermis maintains tissue homeostasis. Basal cells in the epidermis are mitotically active but initiate terminal differentiation by detaching from the basement membrane, withdrawing from the cell cycle, and migrating upward to the skin surface in a columnar fashion. When these cells divide to produce committed progenitor cells, they eventually become terminally differentiated and move to the skin’s surface. Through terminal differentiation, these cells progress through three distinct stages in which they become the three layers of the epidermis: the spinous layer, followed by the granular layer, and finally, the keratinocytes lose their nuclei to become the stratus corneum. These cells must be continuously generated throughout life to maintain a protective barrier, as stratum corneum cells are constantly shed. According to Weinstein et al., the process of epidermal regeneration occurs at an average of 39 day cycles throughout life. This group averaged that the mean transit times through the three layers of the epidermis are as follows: 13 days, 12 days, and 14 days for the spinous, granular, and stratus corneum layers, respectively.

Several molecular signaling pathways necessary for epidermal differentiation, stratification, and acquisition of barrier function have been identified, including Notch, mitogen-activated protein kinase, nuclear factor-κB, p63, the AP2 family, CCAAT/enhancer-binding protein transcriptional regulators, interferon regulatory factor 6, grainyhead-like 3, and Kruppel-like factor 4.

Notch and p63 signaling pathways have been found to be critical in epithelial terminal differentiation. These pathways dictate the switch from basal cells to spinous layer cells, as has been demonstrated in gain and loss of function in vertebrates. Senoo et al. studied p63 null mice during embryogenesis to determine whether p63 was necessary for epidermal formation from primitive ectoderm. This group noted that mice lacking p63 had sparse areas of epidermis in comparison to wild type embryos, which showed a continuous and well-formed epidermis. Likewise, through conditional ablation of RBPJ (a DNA-binding protein essential to the Notch pathway) and knockout studies on Hes1 (one of the main epidermal Notch target genes), Notch has been determined to be essential to the conversion of basal cells to spinous cells.
The hair follicle is an appendage of the mammalian epidermis that is composed of an external outer root sheath attached to the basal lamina and contiguous with epidermis, a channel, and a hair shaft. The hair follicle and its sebaceous gland together are called the pilosebaceous unit. The base, or bulb, of the hair follicle contains committed but proliferating progenitor cells or matrix encasing the dermal papilla, which are specialized mesenchymal cells. The hair and its channel grow from this region (Fig. 3).

Fig. 3. Epithelial stem cells. Epithelial stem cells are located in the bulge area of the hair follicle, in sebaceous glands, and in the lower layer of the epidermis. This figure shows the three layers of the epidermis: the spinous layer, the granular layer, and the stratum corneum. During regeneration, cells migrate vertically upward to the stratum corneum, where they flatten and act briefly as a physical barrier. These cells are then sloughed off.

The hair follicle is an appendage of the mammalian epidermis that is composed of an external outer root sheath attached to the basal lamina and contiguous with epidermis, a channel, and a hair shaft. The hair follicle and its sebaceous gland together are called the pilosebaceous unit. The base, or bulb, of the hair follicle contains committed but proliferating progenitor cells or matrix encasing the dermal papilla, which are specialized mesenchymal cells. The hair and its channel grow from this region (Fig. 3).

Two thirds of the pilosebaceous unit contributes to the hair cycle, which is a process with three stages: catagen (or degeneration), telogen (the rest phase), and anagen (growth). In conditions supporting apoptosis,
the hair follicle proceeds through catagen and telogen, during which time hair growth ceases, the lower portion of each follicle degenerates, and a rest period ensues. After the rest period, the dermal papilla initiates regeneration (anagen onset) and the production of a new hair from follicle epithelial cells at the follicle’s base. This new follicle develops immediately adjacent to the original follicle, and a bulge is created in the external outer root sheath as regenerating cell progenitors reorganize. This bulge, which develops early on in skin development, is now a well-known and ideal epithelial stem cell niche, existing in a protective, well-vascularized, innervated area.32

Ito et al. used fate mapping experiments to study the contribution of bulge stem cells to epidermal repair after injury. The data from these experiments showed that, after the epidermis was injured, cells from the bulge region of the hair follicle initially migrated to the wound and acquired an epidermal phenotype, thereby assisting in wound healing. However, these cells are thought to be transient amplifying cells, cells that are short-lived, initially responding to injury but sloughed within two weeks. The data are notable in that they distinguish epidermal and bulge cells from each other but also show that bulge cells are able to repopulate the interfollicular epidermis in response to stress.33 Experiments performed by Levy et al. confirm these findings.34,35 Furthermore, Oshima et al. showed, through transplantation of the bulge region, that bulge cells were capable of repopulating the epidermis, sebaceous gland, and all epithelial layers of the hair follicle.36

There are a number of stem cell niches in the epidermis, and it is now thought that two described above — the interfollicular epidermis and the bulge region of the hair follicle — can supply each other when damaged. This idea is clinically important in that the skin, the largest organ of the body, may be similar to bone marrow as a stem cell reservoir.

Adult stem cells in the human epithelium are responsible for maintaining tissue homeostasis and responding to injury. As previously mentioned, early gestation fetal skin wounds have the ability to heal without scarring, while adult wounds heal with a scar.8 As humans age, their wound healing ability diminishes. If stem cells are presumed to be responsible for cutaneous wound healing, these observations would suggest that adult stem cells are affected by aging.
The effects of aging could negatively affect the quality of stem cell machinery or deplete the number of stem cells available to respond to proliferation signals. In support of this hypothesis, studies of keratinocytes isolated from older human donors display a lower proportion of holoclones in clonogenicity assay in comparison to those from younger donors. Another study showed that epidermal cells from elderly individuals show increased positivity for p16INK4A, a molecule essential for G1 arrest.

3.3 Mesenchymal stem cells

Mesenchymal stem cells (MSC), initially isolated on the basis of their plastic adherence, are non-hematopoietic stromal cells that are capable of differentiating into mesenchymal lineages such as bone, cartilage, muscle, and fat. Although they are present as a rare population of cells in bone marrow, representing 0.001% of the nucleated cells, they are expandable in culture while retaining their growth potential and multipotency. MSCs have been isolated from various sites other than the bone marrow, including adipose tissue and amniotic fluid, and show phenotypic heterogeneity. Because MSCs can be expanded in culture with relative ease and differentiated into several tissue types in vitro, MSCs represent a promising source of stem cells for wound repair. Moreover, since MSCs are isolated from adult tissues, the use of these cells could avoid some of the obstacles associated with the use of embryonic stem cells. For example, if adult stem cells are transplanted back into the patient from whom they are harvested, these cells should avoid rejection by the immune system.

Several studies show that MSCs have the ability to migrate across endothelial cell layers to reach injured tissue. One group harvested bone marrow cells from enhanced green fluorescent protein (EGFP) transgenic mice and transplanted the cells into lethally irradiated mice via tail vein injection. These mice were then wounded. Many EGFP+ cells were found in both normal cells and at the wound edges. Site-directed delivery of MSCs has shown that these cells can home to a variety of tissues, particularly after injury. Many studies have used injection of allogeneic MSCs into the vascular system to demonstrate that these cells home to various epithelium-lined organs including the lung, gut, skin, and
Several groups have also reported that MSCs can migrate into infarcted myocardium. These experiments demonstrate the multipotency of MSCs and their potential utility in complex tissue repair and regeneration. However, mechanisms by which MSCs home to tissues and migrate across endothelium are not well understood. It is likely that injured tissue expresses specific ligands or receptors to recruit MSC into the site of injury, similar to leukocyte trafficking, adhesion, and infiltration to site of inflammation.

Increasing evidence has shown that MSCs participate in cutaneous wound healing. During injury repair, MSCs can differentiate and then directly participate in the structural repair of a wound, or use paracrine signaling with secreted factors to support wound healing and modulate the immune system. For example, when MSCs were transplanted on the surface of deep burn wounds in rats, there was an acceleration of the formation of new vessels and granulation tissue along with a decrease in inflammatory cell infiltration. Also, application of MSC to cutaneous wounds led to accelerated closure in an excisional wound splinting model in both normal and diabetic mice. MSCs were found to express keratinocyte-specific markers and high levels of vascular endothelial growth factor (VEGF) and angiopoietin-1, suggesting that MSCs promoted wound healing by differentiation and release of pro-angiogenic factors. Another study demonstrated that MSCs transdifferentiated into keratinocytes, endothelial cells, and pericytes in the wounded skin of mice that received intravenous injection of MSCs. When human bone marrow-derived MSCs were applied to full-thickness skin defects in mice, all skin wounds were reported to heal without a scar or retraction. All these findings from animal transplantation studies demonstrate that MSCs can contribute to the repair of injured skin and can serve as the cell source for regenerative therapy.

There are already some encouraging results from human studies utilizing MSC as a therapeutic agent for tissue repair. In a study of chronic non-healing wounds that failed conventional therapy, application of autologous bone marrow cells led to complete closure with evidence of dermal rebuilding with less scar. More recently, the application of fibrin spray containing autologous bone marrow-derived MSCs produced accelerated wound closure in both excisional and chronic wounds.
Despite rapid progress in evaluating the efficacy of MSC transplantation on wound healing, many questions still need to be addressed. Because of the crude method of isolating MSCs, uncertainties remain with respect to the defining characteristics of these cells, such as specific markers to identify and trace the lineage of resident MSCs. A recent hypothesis is that MSCs are pericytes, a supporting cell for blood vessels. This hypothesis raises an interesting connection between these cells and angiogenesis, a key component in wound repair. More research is needed to extensively study not only MSCs, but also other cells and factors that make up the microenvironment or niche that supports the survival and differentiation of the cells. Further studies characterizing the MSC and its niche would help to determine the extent to which MSCs act as stem cells versus as sources of secreted factors. This research would also allow dissection of this heterogeneous cell type into more distinct and functional subpopulations.

### 3.4 Induced pluripotent stem cells

The reprogramming of adult cells to a pluripotent state is one of the most exciting recent advances in stem cell biology. Takahashi and Yamanaka published a landmark paper in 2006 that defined a specific set of transcription factors capable of reverting differentiated cells back into a pluripotent state, thus creating “induced” pluripotent stem (iPS) cells. Through screening of 24 pre-selected ES-cell specific factors in the murine system, four transcription factors — Oct4, Sox2, Klf4, and c-Myc — were found to be sufficient to reprogram adult mouse fibroblasts into ES-like iPS cells. The same combination of transcription factors has been demonstrated to be sufficient for pluripotent induction of human cells as well. Mouse and human iPS cells closely resemble molecular and developmental features of blastocyst-derived ES cells. Different research groups have shown that iPS cells injected into immunodeficient mice give rise to teratomas comprising all three embryonic germ layers, similar to ES cells. In addition, when injected into blastocysts, iPS cells generated viable high-contribution chimeras (mice that show major tissue contribution of the injected iPS cells in the host mouse) and contributed to the germline. Furthermore, various studies have shown that iPS cells express key ES cell markers.
The reprogramming of adult cells to ES cell-like pluripotent states provides new exciting possibilities. iPS technology can be used to generate patient-specific cell lines for therapeutic approaches, eliminating the concern for immune rejection or bioethical concerns associated with use of ES cells. However, multiple issues need to be addressed before this technology can be used in patients. iPS cells are generated with the use of retroviral and lentiviral vectors to activate the necessary reprogramming transcription factors. Because of viral integration, the risk of insertion mutagenesis could lead to uncontrolled modification of the genome.\textsuperscript{61} Much progress has been made recently in generating integration-free murine iPS cells, and various studies using adenoviral, plasmid-based, and recombinant protein-based strategies have reported that viral integration is not required for the reprogramming process.\textsuperscript{62–64} The issue of viral integration aside, the safety of iPS cells needs to be vigorously tested because all essential reprogramming factors are oncogenes that, if overexpressed, can lead to cancer.\textsuperscript{65} Chimeras and progeny mice derived from iPS cells had higher than normal rates of tumor formation than those derived from ES cells, which in some cases may be due to reactivation of the transfected c-Myc oncogene.\textsuperscript{66} These key issues need to be further elucidated to define the safety of iPS cell use in regenerative medicine.

4. Conclusion

The process of wound healing is one of the most complex biological processes, influenced by numerous secreted factors including growth factors, cytokines, and chemokines. Complications in wound healing such as inadequate healing or hypertrophic and keloid scars can arise from abnormalities in the repair process. Systemic and local influences such as malnutrition and infection can impair the normal wound repair process. Even without such harmful factors, most injuries in adult human result in deposition of non-functioning fibrotic tissue.

Although tremendous progress has been made in our understanding of numerous factors involved in wound healing process, these findings have not yet led to substantial advances in patient care. The cellular organizer of the wound repair process is still not well understood. One method to
address both underhealing and overhealing would be to manipulate the key cell types that modulate this overall process. Stem cells have enormous potential to fulfill this role, as the cells could both coordinate their actions with the immune system and mediate regeneration of lost tissue to promote an ideal wound repair outcome.

Recent advances in stem cell research offer promising opportunities for regenerative medicine. Diverse cell types including ES cells, MSC, resident tissue stem cells (such as epithelial stem cells), and iPS cells are currently under intense investigation. Novel cell-based therapies using stem cells to induce tissue regeneration hold great promise for wound repair and modern medicine.

References


1. Introduction

This chapter begins by reviewing the structural and compositional make up of articular cartilage followed by a brief discussion of osteoarthritis. Defects of cartilage that may lead to osteoarthritis are discussed along with associated surgical strategies for repair. The authors will also describe numerous tissue engineering strategies aimed to repair cartilage defects including materials and small molecules that direct stem cell differentiation towards a chondrogenic lineage. The goal of this chapter is to not be all encompassing on each of these areas but to give an overview to the reads of the exciting research being performed in the area of cartilage tissue engineering and the current limitations.

2. Structure of Articular Cartilage of the Knee

Articular cartilage lines the surface of all diarthroidal joints including the hips, knees and shoulders. The diarthrodial joints are enclosed in a fibrous capsule. Lining the inner surface of the capsule is the synovium which
contains synoviocytes. The synoviocytes secrete the synovial fluid that provides the nutrients to the articular cartilage along with lubrication. Due to the smooth surface of the articular cartilage and the synovial fluid, minimal friction is exerted in the joint space during normal motion.¹

Articular cartilage is predominantly composed of water and extracellular matrix (ECM). Additionally, a single cell population resides within the articular cartilage, known as chondrocytes, which emerge from mesenchymal precursor cells during limb development.² The ECM is divided into two major categories, collagen and proteoglycans (PGs). The main type of collagen in articular cartilage is type II and to a lesser extent type IX and XI.³ Collagen fibers are found throughout the cartilage and contribute to the tensile strength of the tissue.¹ Proteoglycans are composed of a core protein, typically aggrecan, with glycosaminoglycans (GAGs) bound to a serine residue via a trisaccharide linker. The predominant GAG molecules of articular cartilage are chondroitin sulfate and keratin sulfate. Glycosaminoglycans are polysaccharides of which most sugar moiety contains one or multiple negative charge(s) resulting in elongation of the PGs. Aggregates of PGs are formed when multiple PGs bind to hyaluronic acid, a non-sulfated GAG molecule, via link proteins. Moreover, due to the highly negative charge the molecules are extremely hydrophilic and can trap in large amounts of water resulting in a highly elastic tissue.¹ The collagen network, with its high tensile strength, interacts with the PGs resulting in a fiber reinforced composite with high compressive strength.

Similar to other connective tissue, articular cartilage functions via its ECM, offering its biological function and mechanical integrity. The resident chondrocytes play a key role in ECM production and turnover. They serve to produce the collagen and PGs of cartilage along with the molecules that remodel the matrix, specifically matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) which predominantly degrade collagens and aggrecans, respectively. The anabolic and catabolic nature of chondrocytes results in natural cartilage turnover. An imbalance in the natural cartilage homoeostasis leads to arthritis.

There are four zones in articular cartilage, with the superficial tangential zone containing flattened chondrocytes. Collagen and cells of this layer
are aligned tangential to the surface. This layer has been suggested to contain progenitor cells. Next is the transition layer, where the chondrocytes and collagen are randomly oriented followed by the deep zone. Collagen and cells of the deep zone are aligned perpendicular to the surface. A smooth transition from articular cartilage to subchondral bone is marked by calcified cartilage starting at the tidemark. The tidemark is the basophilic line that separates the calcified and uncalcified cartilage. Due to the spatial orientation of the ECM and cells along with specialized mechanical properties, it is particularly difficult to engineer the full thickness of articular cartilage.

Articular cartilage is avascular, acquiring most of its nutrients from the synovial fluid. Because of the dense ECM which limits chondrocyte mobility, and the avascular nature, cartilage has a limited ability to self-repair. This is problematic when articular cartilage is damaged due to trauma or osteoarthritis making it an excellent candidate for tissue engineering strategies. The exact mechanism that triggers OA is not well understood, however, when patients have cartilage lesions caused by some form of trauma they are at higher risk of developing OA. In the United States alone, 27 million people live with OA.

3. Osteoarthritis of the Knee

Osteoarthritis (OA) is a disease of the whole organ system, including the synovium, subchondral bone and articular cartilage. It is unknown which pathological changes initiate OA. However, in OA there is an imbalance in the rebuilding and degradation of the ECM resulting in a net loss of cartilage. Radiographic changes of the articular cartilage include thinning of the joint space and sclerosis of the subchondral bone. Histologically, a loss of proteoglycans, or fixed charges (via safranin-O or toluidine blue staining) is observed. A loss in total collagen occurs at later stages of OA, however, it is preceded by increased swelling properties of the cartilage due to loosening of the collagen fibers seen at initial stages of OA. The first stages of OA are marked by fibrillation of the cartilage surface; proceeding to complete loss of articular cartilage such that the subchondral bone is exposed. The tidemark is also seen to thicken which correlates to calcification of the deep zone, known as hypertrophy, and marked with an
increase in type X collagen. Articular chondrocytes are seen to be clustered, as opposed to single cells, due to initiation of proliferation. Bony growths, known as osteophytes, can also be found.

The two major classes of degradation molecules occurring naturally in articular cartilage are increased in OA. A disintegrin and metalloproteinase with thrombospondin motifs, specifically ADAMTS-4 and -5, play a crucial role in the degradation of aggrecan. Due to degradation of aggrecan, the PGs disassemble and diffuse out of the articular cartilage, resulting in a decrease in compressive strength due to reduction of negative charge. Matrix metalloproteinases play a key role in the degradation of collagen and gelatin. The three main MMPs prevalent in OA are MMP-2, -9 and -13. MMP-13 is a collagenase efficient at degrading type II collagen fibrils. Gelatinases, MMP-2 and -9, follow up by degrading the newly formed gelatin. Both classes of enzymes play a pivotal role in matrix degradation that causes a reduction in the mechanical integrity of articular cartilage. They also pose a particular challenge when attempting to engineer the tissue as they may serve to inhibit the fill tissue formation — an aspect not typically modeled during in vitro testing of tissue engineering strategies.

Inflammation also plays an important role in cartilage degradation and the occurrence of OA. Specifically, interleukin-1 (IL-1) and tumor necrosis factor (TNF) are inflammatory cytokines found to be increased in primary cultures of OA chondrocytes compared to healthy chondrocytes. IL-1β and TNF-α have been found to be localized to the superficial zone of articular cartilage. These cytokines induce the production of the protease along with proinflammatory cytokines acting as a positive feedback loop. Additionally, these cytokines are known to be associated with a decrease in chondrocytic gene. Both the increase in proteases and decrease in terminally differentiated chondrocytic gene expression contribute to the reduction in ECM.

These cytokines are also known to activate many intracellular signaling pathways including JNK, p38 MAPK, ERK and NF-κB. The abundant form of NF-κB is a heterodimer of p65 and p50. It is sequestered in the cytoplasm via interacting with inhibitor of κB (IκB, either α or β). The NF-κB heterodimer is released from IκB after IκB is phosphorylated and targeted for ubiquitin mediated degradation. This allows for NF-κB
translocation to the nucleus where it transcriptionally regulates many cytokines including proinflammatory cytokines IL-1 and TNF-\(\alpha\), and expression of I\(\kappa\)B-\(\alpha\) along with further downregulation of chondrocyte-specific genes.

Patient symptoms associated with OA include pain and instability which can result in immobility. Pain is felt in early OA coinciding with activity level. However, as OA progresses, chronic pain may become more persistent, even in the absence of activity.\(^{15}\) The underlying cause of pain with OA is not fully understood, as articular cartilage is aneural. However, some have theorized the pain resides from swelling of the synovium, in turn affecting the peripheral nervous system.\(^{11}\) Also, MRI imaging has shown lesions in the bone marrow which is better associated with patients experiencing pain than those who do not.\(^{16}\) Non-surgical treatments for pain management include non-steroidal anti-inflammatory drugs (NSAIDS) and intra-articular injections of hyaluronic acid or corticosteroids.

4. Surgical Strategies for Repairing Focal Cartilage Defects

Focal cartilage defects can occur due to trauma, repetitive impact or progressive mechanical degeneration. These defects, if left untreated, may result in further knee degeneration leading the osteoarthritis. There are three types of cartilage defects: (1) partial thickness, (2) full thickness and (3) osteochondral which penetrate into the subchondral bone.\(^{17}\) Occurrence of OA appears on average ten years sooner in patients with cartilage defects. Because cartilage has a limited ability to repair itself surgical techniques are employed to repair the defect site by filling with biological material to reduce or eliminate the progression to OA.

Surgical treatments for OA are only employed when non-surgical treatments have failed. There are three primary techniques utilizing to repair cartilage defects: (1) bone marrow stimulation, (2) mosaicplasty or osteochondral autograft transfer system (OATS) and (3) autologous chondrocyte implantation (ACI). All of these procedures result in inferior fibrocartilage formation and mechanical characteristics. This is due to the disorganization of the fill tissue, predominantly composed of type I collagen, whereas articular cartilage has organized cellularity and ECM.
4.1 Bone marrow stimulation

Bone marrow stimulation utilizes natural repair strategies to fill cartilage lesions. The microfracture technique is the most widely used.\textsuperscript{18} This technique involves debridement of the defect area down to the subchondral bone followed by creating multiple punctures into the subchondral bone to allow bone marrow to come into the defect site.\textsuperscript{18} A fibrin clot will form within the defect which contains mesenchymal stem cells and acts to facilitate migration of additional mesenchymal stem cells within days.\textsuperscript{19} These cells will then go on to form new fibrocartilage tissue. Positive clinical outcomes include reduction in pain. However, results are found to deteriorate within 18 to 24 months post-surgery.\textsuperscript{20,21}

4.2 Mosaicplasty and osteochondral autograft transfer system (OATS)

Mosaicplasty and OATS procedures employ osteochondral plugs transplanted into the defect site. They are suitable for smaller size defects (less than 4 cm\textsuperscript{2}) due to surgical challenges and donor site morbidity.\textsuperscript{22,23} One large graft, or in the case of mosaicplasty multiple small grafts about 1 mm in diameter, is isolated from a non-load bearing area of the knee and transplanted to the defect site. The implanted graft maintains its articular cartilage structure. However, the bonding tissue between the implants is fibrocartilage in nature.

4.3 Autologous chondrocyte implantation

The final surgical procedure for cartilage repair is autologous chondrocyte implantation (ACI).\textsuperscript{24} This is a two-step process. In the first stage cartilage from a non-load bearing area is removed. The chondrocytes are then isolated from the ECM and expanded \textit{in vitro}. Expanded cells are then reimplanted during the second surgery into the focal defect and held into place with a periosteal flap. The periosteal flap is believed to play a role in defect repair due to a paracrine effect or may serve as a source for autogenous cells.\textsuperscript{25} This technique has been found to have comparable results to microfracture after two years.\textsuperscript{26} In addition, histological evaluation of
the fill tissue has shown some hyaline-like cartilage with the rest being fibrocartilage. However, many limitations to this procedure exist, including donor site morbidity, limited cell supply and dedifferentiation of the isolated cells. Dedifferentiation occurs due to \textit{in vitro} expansion in monolayer culture resulting in loss of cartilage specific gene expression and ECM production. Redifferentiation of the expanded chondrocytes is necessary in order to obtain hyaline-like cartilage.

5. Scaffolds for Assisting Operative Techniques

Multiple scaffolds have been generated to attempt to enhance the regenerating cartilage. Materials have been utilized to anchor the cells in place and augment the operative procedures. Using tissue engineering strategies to augment operation procedures is of interest to the research community because it offers many opportunities to direct the fill tissue. Specifically, materials have been utilized to direct redifferentiation of chondrocytes, differentiation of stem cells and fill tissue architecture to match that of native tissue. Cell-free and cell-laden scaffolds have been used in the clinical setting. Cell-laden scaffolds are homogenous in cell distribution, better recapitulate the native three-dimensional environment and help to maintain or induce the chondrocytic phenotype.

5.1 Collagen scaffolds for augmenting ACI

Multiple collagen scaffolds have been developed and used clinically for augmenting the ACI procedure. A type I/III collagen flap has been designed to eliminate the need for periosteal flaps. It had significant advantages compared to the original technique, including less invasive surgery, reduced surgery time and decreased postoperative pain. This technique was limited in that suturing of the collagen membrane to the articular surface is required and tedious.

Due to enhanced histological findings with the collagen flap a new technology was developed to eliminate the limitation of the collagen flap, a procedure known as matrix-induced autologous chondrocyte implantation (MACI\textsuperscript{®}). As opposed to injecting cells underneath the flap, cells are seeded directly onto a type I/III collagen membrane
isolated from porcine peritoneal cavity. The cell-laden collagen membrane is then placed into the prepared defect using a thin layer of fibrin glue to secure it to the defect. The chondrocytes can penetrate into both the collagen membrane and the fibrin glue and retain their chondrocytic phenotype. The resulting tissue is hyaline-like and positive for type II collagen. Similar results are seen with a porcine type I/III bilayer matrix, Chondro-Gide®.

Another ACI augmenting collagen scaffold, NeoCart®, is a three-dimensional type I collagen scaffold seeded with expanded chondrocytes. The seeded scaffold is then cultured in a bioreactor. The total time for implant development is 67 ± 18 days. A proprietary tissue adhesive, CT3 (Histogenics), composed of collagen and polyethylene glycol is used to secure the implant in place. Since this implant is fairly new, minimal clinical outcomes have been observed. However, the treated patient’s pain score was lower than at baseline. Also, range of motion and knee function was seen to be improved.

5.2 Collagen scaffolds with autologous mesenchymal stem cells

Mesenchymal stem cells are a promising cell source for tissue engineering because they have the ability to differentiate into multiple lineages including bone, cartilage, fat and astrocytes. They can be isolated from individual patients in a minimally invasive manner and expanded in vitro without losing their ability to differentiate. Mesenchymal stem cells have been evaluated clinically for their ability to augment bone marrow stimulation. In this work, 2 mm of the subchondral bone was removed until bleeding was seen. Perforation was then performed using 1.2 mm Kirshner wire to facilitate further bleeding. MSCs were previously isolated using standard procedures and expanded through one passage in vitro. The day before surgery, MSCs were lifted from the tissue culture plates (average 13 million) and embedding into 1.2 ml of 0.25% type I collagen from porcine tendon. The cell suspension was seeded onto collagen sheets (derived from bovine source) and allowed to gel. The collagen/MSC scaffold was cultured overnight in DMEM supplemented with autologous serum and antibiotics. The composite
was placed into the defect with the collagen sheet covering the upper side. A peristium flap was used to secure the material in place. Control patients received the collagen scaffold with no cells and others did not receive the perforation technique. The resulting fill tissue was found to be mechanically weaker in all groups compared to the surround cartilage. However, histologically (using toluidine blue) the cell-laden group was superior to the cell-free group, exhibiting a metachromatic staining and some hyaline-like tissue. Further work is necessary to understand the origin of the cell population residing within the scaffolds after implantation.

5.3 Hyaluronic acid (HA) scaffolds

Hyaluronan is found in all soft tissues. Hyaluronan is an unsulfated glycosaminoglycan of disaccharide repeat units, glucuronic acid and N-acetylglucosamine. Its molecular weight ranges from 4000 to $8 \times 10^6$ Da. As mentioned previously, it interacts with proteoglycans along with other proteins and molecules. In addition, cells expressing CD-44 can bind to and migrate on HA. Degradation products of HA contribute to many biological activities including size-dependent affect on chondrogenic differentiation, vascularization and angiogenesis.

Modified HA has been used as a scaffold for articular cartilage defect repair, specifically Hyaff®. Hyaff® hyaluronic acid starts as a molecular weight of 180–200 kDa. Esterification of the glucoronic acid groups is performed. By varying the alcohol used in the esterification reaction and the degree of substitution bioresorbabilty, water solubility and residence time can be varied.

HYAFF® 11 is biocompatible and resorbable without the presence of an inflammatory response. It has been used to augment the ACI procedure. Isolated chondrocytes were expanded in vitro followed by seeding and culturing on the scaffold for an additional two weeks. Used in this way, it is marketed as Hyalograft® C. In animal models, these scaffolds have been shown to develop hyaline-like cartilage and integrate with the surround tissue. Clinical trials have shown that patients treated with Hyalograft® C had significant improvements in pain, physical activity and knee function.
5.4 Fibrin scaffolds

Fibrin glue is a material that synthetically mimics fibrin clots, part of natural tissue repair after injury. It is a two-component system composed of fibrinogen and thrombin. Once mixed together, the thrombin degrades fibrinogen to fibrin which acts as a tissue adhesive and three-dimensional scaffold. Fibrin glue is used as an adhesive to secure various scaffolds into cartilage defects due to its chondroinductive property.\textsuperscript{41}

Scaffolds of fibrin can also be made and have been studied for cartilage tissue engineering.\textsuperscript{42} Fibrin scaffolds have been used clinically, again, to augment the ACI procedure. In this treatment, the expanded chondrocytes are mixed with fibrinogen and thrombin to form a cell-seeded fibrin scaffold. The defect site is then debrided to the subchondral bone and the subchondral bone penetrated. A thin layer of fibrin glue is applied followed by molding and implantation of the cell-seeded fibrin scaffold. Another layer of fibrin glue is then applied. This technique is superior to ACI alone because it eliminates the need for a periostea flap, reducing patient recovery time.\textsuperscript{43}

Zimmer Inc. has also developed a product using fibrin glue.\textsuperscript{44} The product, known as DeNovo\textsuperscript{®} NT Graft, utilizes mince cartilage tissue from allogous juvenile cartilage. Viable cartilage pieces are mixed intra-operatively with fibrin and placed into the defect site. A thin layer of fibrin is then applied over the implant to maintain placement. Utilizing viable tissue results in a cell source that can migrate out of the minced pieces and fill the defect area. Advantages over the ACI procedure include only requiring a single operation and eliminating the need for costly \textit{in vitro} expansion.

5.5 Chitosan scaffold

Chitosan is a polysaccharide derived from deacetylating chitin isolated from the exoskeleton of crustaceans. It is composed of D-glucosamine and N-acetyl-D-glucosamine linked via a $\beta [1-4]$ linkage. Due to its positive charge it behaves as a bioadhesive and can adhere to negatively charged tissue.\textsuperscript{45} BST-CarGel\textsuperscript{®} is composed of a mixture of chitosan and uncoagulated whole blood which gels within ten minutes. BST-CarGel\textsuperscript{®}
has been evaluated for its ability to augment microfracture technique.\textsuperscript{46} First, the microfracture technique is performed and a “dry field” is created. At least 5 ml of peripheral blood is obtained, 4.5 ml of which is mixed with the supplied BST-CarGel\textsuperscript{®}. This mixture is then placed within the prepared cartilage defect and allowed to gel for 15 minutes. Multiple animal studies have been performed and have shown that using BST-CarGel\textsuperscript{®} resulted in increased hyaline-like cartilage formation, GAG content, collagen content and repair tissue volume when compared to microfracture alone.\textsuperscript{46} Clinical studies have shown that BST-CarGel\textsuperscript{®} decreased pain and stiffness along with increased joint function.

### 5.6 Polyester-based scaffolds

Polyesters are synthetic polymers of repeating degradable ester groups with various amounts of carbons separating the esters and side chains. For example, poly(lactic acid) and poly(glycolic acid) contain one carbon between the ester bonds with poly(lactic acid) containing a methyl group on the $\alpha$-carbon. Another common polyester is poly($\varepsilon$-caprolactone) with contains six carbons between the ester groups. Polydioxanone is a polyester-ether containing three carbons along with oxygen (making the ether) between each ester bond. The esters can be hydrolytically degraded to carboxylic acids and alcohols. Degradation rate of polyesters can be controlled by the specific polyester used, copolymerization or mixing multiple types of polyesters together. These variations change the degradation rate due to altering the degree of crystallinity. The range of degradation that can be achieved is a couple of weeks up to years.

One example clinically evaluated is a polymer-based scaffold of polylactic/polyglycolic acid (polyglactin, vicryl) and polydioxanone collagen fleece coined Bioseed\textsuperscript{®}-C.\textsuperscript{47,48} Chondrocytes isolated and expanded from the patient are seeded within the scaffold and secured using fibrin. The scaffold is cut to the shape of the defect, armed with vicryl sutures at each corning and secured using K-wires passed through the femur. A press fitting technique is used to securely hold the Bioseed\textsuperscript{®}-C scaffold in place. This technique is more stable than ACI and eliminates donor site morbidity associated with periosteal flaps. The procedure is also performed completely arthroscopically so it reduces adhesions associated with open surgeries.
However, in a comparative clinical study between ACI and Bioseed\textsuperscript{\textregistered}-C no differences were seen in the clinical outcomes between the treatment groups.\textsuperscript{47}

Another polyester system undergoing clinical evaluation utilizes mince cartilage pieces collected from the patient seeded onto a foam scaffold coined cartilage autograft implantation system (CAIS).\textsuperscript{44} The mince pieces are held into place with the use of fibrin adhesive. The scaffold is then stapled into place. Evaluation of a similar scaffold in goats after six months of implantation resulted in completely filled defects with hyaline-like cartilage that had more type II collagen and less type I collagen than scaffolds without minced cartilage.\textsuperscript{49}

6. Mesenchymal Stem Cells for Cartilage Tissue Engineering

Limitations exist in using articular chondrocytes. First, cells must be isolated from the donor site during the initial surgery which results in two invasive procedures in order to repair the defected cartilage. Second, the number of chondrocytes that can be isolated is limited and therefore the cells must be expanded \textit{in vitro}. This results in a costly procedure and dedifferentiation of the chondrocytes due to cell expansion.\textsuperscript{50} Finally, donor site morbidity is of concern and limited research has been done to investigate these effects.

Alternative cell sources exist for cartilage tissue engineering. These include embryonic stem cells and mesenchymal stem cells. Embryonic stem cells are derived from the inner cell mass of an embryo. Due to the isolation technique, ethical issues surrounding embryonic stem cells have limited the research and extent of use of these cells. Alternatively, mesenchymal stem cells can be isolated from bone marrow, umbilical cord blood, adipose tissue, synovial cells, and peripheral blood.\textsuperscript{51} The first three are the most common sources under investigation. Bone marrow-derived MSCs have been studied more extensively than any other source. Umbilical cord blood-derived MSCs are limited by availability because they can only be isolated at birth. Adipose-derived MSCs can easily be isolated from lipoaspirate after cosmetic liposuction. This derivation technique has proven to result in higher cell yields than that of bone
marrow and umbilical cord-derived MSCs. However, this cell source is limited to individuals with excess fat to isolate cells from. Human adipose-derived MSCs have also been shown to have an inferior ability to differentiation towards cartilage and bone when compared to human bone marrow-derived MSCs.

Differentiation of MSCs towards a chondrogenic lineage can be induced by a variety of physical and chemical factors. A three-dimensional culturing system is required in order for adequate chondrogenesis to occur. Examples of three-dimensional environments used are pellet culture, high density culture, hydrogels and sponge-like scaffolds. Chemical factors are included into the medium to elicit more specific differentiation. Dexamethasone is supplemented into the medium though the exact mechanism of how it induces chondrogenesis is not well understood. It is believed to increase Sox-9 gene expression, a transcription factor that activates type II procollagen gene expression. Various growth factors and proteins are also supplemented into the medium to induce differentiation including transforming growth factor (TGF)-β1 and -β3, IGF-1, bone morphogenic proteins (BMPs) and fibroblast growth factor (FGF)-2. These factors are also known to be key regulators of chondrogenesis during skeletal development.

7. Hydrogels for Directed Differentiation of Mesenchymal Stem Cells

Hydrogels have been used as a three-dimensional scaffold for studying cellular function and differentiation. As mentioned previously, bioactive materials have been used as scaffolds. Many bioinert materials have been studied as a means to encapsulate cells in a three-dimensional configuration. Examples of bioinert hydrogels include poly(ethylene glycol)-diacrylate (PEGDA), poly(vinyl alcohol) (PVA) and alginate. As a result the soluble components contained in the medium are the primary contributors to directing cell fate. In the case of bioactive hydrogels, the cells can interact with the substrate resulting in materials that can be used to direct cellular activity. When encapsulating MSCs into hydrogels it is of particular interest to engineer materials that direct differentiation. In
this way, scaffold assisted differentiation occurs as a result of the material-cell interaction; resulting in a scaffold with potential to enhance \textit{in vivo} differentiation, where factors contained in medium are not available.

### 7.1 Functionalized Poly(ethylene Glycol) Hydrogels

Poly(ethylene glycol) hydrogels have been investigated extensively as bioinert scaffolds for three-dimensional cell culture. Poly(ethylene glycol) has been functionalized with acrylate groups to facility crosslinking via numerous methods including ultraviolet light exposure and a free radical initiator, reduction-oxidation reaction, and Michael addition.\textsuperscript{55} They have been shown to support differentiation of MSCs towards a chondrogenic lineage via the factors present within the medium.

Numerous research groups have functionalized PEG hydrogels with RGD (arginine-glycine-aspartic acid), a cell-binding peptide found in many proteins. When anchorage-dependent cells are encapsulated into inert three-dimensional hydrogels adhesion is not possible resulting in a large percentage of apoptotic cells. Therefore, incorporating adhesion peptides, such as RGD, facilitates cell survival within the hydrogels. Nuttelman and colleagues\textsuperscript{56} demonstrated that human MSCs survival in PEG hydrogels increases from 15% to 75% when RGD was covalently incorporated into the network. Enhancing cellular survival is of particular interest because tissue formation will be facilitated within the scaffold due to an increase in total extracellular matrix deposition.

When evaluating human embryoid body-derived MSCs (MSCs derived from embryonic stem cells) Hwang and colleagues\textsuperscript{57} found a significant increase in chondrogenesis in the presence of covalently linked RGD in PEG hydrogels. Specifically, increase in GAG and total collagen production was seen on a per cell basis when compared to PEG only hydrogels. This was confirmed by increased safranin-O and type I and II collagen staining. Though favorable to have an increase in type II collagen, type I collagen is less favorable. However, when gene expression was evaluated, type II collagen, aggrecan and link protein increased in the RGD group; whereas type I collagen gene expression was similar to that of PEG hydrogels alone.
Interestingly, when alginate hydrogels were functionalized with RGD chondrogenesis of bovine MSCs was inhibited in a concentration dependent manner.\textsuperscript{58} Similar inhibition of sulfated GAG production was observed in bovine chondrocytes encapsulated in alginate hydrogels with covalently link RGD.\textsuperscript{59} Silk scaffolds covalently linked with RGD also exhibited similar inhibitor effects on chondrogenesis of human MSC.\textsuperscript{60}

Still RGD peptide does have a positive effect on cell viability. To combat the negative effect on chondrogenesis, Salinas and colleagues developed an RGD containing peptide with an MMP-13 cleavage site.\textsuperscript{61} Matrix metalloproteinase-13 activity was observed to peak at an intermediate culturing time when MSCs were undergoing chondrogenesis which resulted in cleavage of the RGD sequence. In this way, the increase in viability can be harnessed earlier followed by removal of the RGD to eliminate the inhibitor effect seen with RGD and chondrogenesis. Though cell viability was seen to decrease at the time at which cleavage was believed to occur, GAG production relative to DNA increased in a time dependent manner even after the RGD sequence was cleaved. In a control group, where the RGD peptide was not cleavable, GAG production decreased supporting the hypothesis that RGD is important for cell viability but necessary to remove to enhance chondrogenesis.

Salinas and colleagues also evaluated a decorin binding peptide, KLER (lysine-leucine-glutamic acid-arginine) in combination with RGD on the chondrogenesis of human MSCs.\textsuperscript{62} Decorin is known to influence fibrillogenesis of collagen at two major sites, RELH (arginine-glutamic acid-leucine-histidine) and KLER. When both RGD and KLER were covalently linked to PEG hydrogels, collagen content increased over the time course of the experiment when compared to RGD and RGD plus scrambled peptide. Gene expression for aggrecan was highest in the RGD/KLER peptide group compared to the controls at early time points, whereas type II collagen expression was highest at later time points.

Another peptide has been evaluated for its effects on chondrogenesis. This peptide, known as collagen-mimetic peptide (CMP), immobilizes collagen via binding through a strand invasion route.\textsuperscript{63} Using a synthetic peptide to bind endogenous collagen is favorable
over collagen scaffolds because it eliminates possible immune response. The material in which the peptide is covalently linked can also be mechanically more robust than collagen gels making them favorable from a practical aspect, also. When compared to PEG hydrogels, CMP containing hydrogels had an increase in cartilage matrix production (GAG and type II collagen) and cartilage-specific gene expression of differentiating goat MSCs. Whereas, type I collagen production, marker for bone formation, and type X collagen gene expression was decreased.

In addition to peptides, GAG molecules can be covalently linked to PEG hydrogels to facilitate enhancement of chondrogenesis. One such GAG molecule, chondroitin sulfate, has been shown to enhance the chondrogenesis of goat MSCs compared to PEG hydrogels. Visualization of the hydrogels after chondrogenesis showed a nodule-like appearance. When investigated further, the MSCs were found to be undergoing a mesenchymal condensation-like state based on gene expression for versican and cadherin 11 (markers found naturally in mesenchymal condensation). CS-based hydrogels also enhanced total collagen production and type II collagen deposition. Also, hypertrophic markers were lower in the CS-based hydrogels. Overall, CS-based hydrogels have the ability to enhance chondrogenesis while reducing hypertrophy and may recapitulate the natural process of mesenchymal condensation.

7.2 Naturally derived materials

Natural derived biomaterials, such as collagen and hyaluronic acid (HA), have been utilized as 3D scaffolds for MSC differentiation. These materials mimic the natural ECM of cartilage and better recapitulate the in vivo environment. Limitations to these types of materials include minimal control over spatial location and orientation of the biomolecule and mechanical weakness. Also, because they are derived from natural sources (i.e. animal tissue) concerns of immune response exist.

Hyaluronic acid hydrogels have been developed via functionalizing HA with methacrylate groups to facilitate free-radical crosslinking. These HA hydrogels have been compared in parallel to PEG hydrogels (with similar mechanical properties). In vitro chondrogenesis of human MSCs
was enhanced in the HA hydrogels based an increase in type II collagen and proteoglycans deposition along with chondrocytic gene expression. In vivo, cartilage specific gene expression was also seen to be higher in HA hydrogels. Three different groups were evaluated in vivo. The group not administered exogenous TGF-β3 or predifferentiated for two weeks in vitro before implantation demonstrated the optimal gene expression profile. This shows that HA hydrogels may be capable to differentiation MSCs towards a chondrocytic lineage without external stimulation.

As mentioned previously, collagen scaffolds have been used in vivo. They have been able to elicit a more hyaline-like cartilage tissue fill favorable for cartilage tissue engineering. A study comparing type I collagen, type II collagen and a bioinert hydrogel, alginate, showed that type II collagen hydrogels enhanced chondrogenesis of bovine MSCs based on gene expression and ECM deposition. Similarly, studies have shown enhancement of osteogenesis in type I collagen scaffolds (type I collagen being the predominant collagen type in bone). These findings support the hypothesis that ECM component found in the native tissue of interest can facilitate enhancement of differentiation towards that tissue lineage.

8. Fiber-Hydrogel Composites

Hydrogels have been the predominant focus for researchers in developing tissue engineering strategies for articular cartilage. Mechanically, they better resemble of the developing limb in comparison to the more stiff mature cartilage. However, they are primitive mimics of articular cartilage as they lack the fibrous nature of native cartilage. Many research groups have investigated methods of producing fibrous scaffolds to act as a physical stimulus similar to that of the fibrous phase of articular cartilage. However, these systems then lack the hydrogel phase. Fewer researchers have investigated fibrous-hydrogel composites for cartilage tissue engineering. These composites employ the use of a hydrogel phase as a delivery vehicle for cells infiltration into the fibrous phase. The hydrogel phase also helps to maintain the round cellular morphology native to cartilage. Fiber-reinforced hydrogels have an added benefit of enhancing the mechanical integrity of the scaffold; this may help in addressing larger size cartilage defects than currently feasible clinically.
Polyesters have been studied extensively as fibrous meshes for cartilage tissue engineering. Favorable results have been obtained for both chondrocytes and chondrogenic differentiation of MSCs seeded directly onto the meshes. Chondrocytes have been seeded onto electrospun poly(ε-caprolactone) meshes and were found to maintain their chondrocytic phenotype.\(^{76}\) Chondrogenic differentiation of MSCs in the presence of TGF-\(\beta\)1 resulted in rounded cell morphology similar to that of native chondrocytes.\(^{77}\) In addition, favorable gene expression was found with chondrogenic MSCs, including increased type II collagen and decreased type X collagen. When compared to pellet culture, chondrogenic MSCs on nanofiber scaffolds were found to have a two-fold increase in GAG production. These findings imply that fibrous polyester scaffolds are good candidates for cartilage tissue engineering. However, limitations exist in high-throughput development of fibrous meshes large enough to fill cartilage defects. Also, cell infiltration into electrospun mats is limited due to the small pore size.

A non-woven fleece of polygalactin, E210, has been implanted subcutaneously in athymic nude mice after infiltrating with alginate containing bovine chondrocytes.\(^{72}\) Crosslinking of the alginate was facilitated by immersing the composites in a calcium chloride solution prior to implantation. After explantation, E210 fleeces without cells and alginate were too weak to evaluate and broke when cut. E210 scaffolds seeded with cells, with and withoutalginate, had similar levels of GAG and type II collagen production. However, the scaffolds with alginate had a more even distribution of cells and did not exhibit any shrinking.

A similar composite scaffold has been evaluated using a PGA mesh and either alginate or type I collagen hydrogels.\(^{73}\) Rabbit MSCs were seeded in a similar manner. At early time significant differences were seen with the alginate-fiber compared to collagen-fiber and fiber-only scaffolds. Cell morphology in the alginate scaffolds was maintained as rounded whereas in the collagen scaffolds a fan-shape was seen. However, the fan-shaped cells became more polygonal over time indicating chondrogenic differentiation. Cell proliferation and differentiation was seen to be delayed in the alginate scaffolds at three weeks. However, after six weeks of culture the GAG content normalized to DNA was significantly higher in the alginate scaffolds compared to collagen scaffolds.
Finally, a woven scaffold composed of PGA yarn has been utilized as a reinforcement material for cartilage tissue engineering. Fibers were oriented in the $x$, $y$, and $z$ directions resulting in a highly interconnected fibrous network where the pore size could be controlled. The hydrogel phase was composed of either agarose or fibrin. The authors were able to show that fibers enhance the mechanical properties of the hydrogels independent of the type of hydrogel used. From a biological perspective, the authors were able to incorporate porcine articular chondrocytes homogeneously throughout the fibrous scaffold with the use of vacuum-assisted infusion. In a later study, chondrogenesis of human adipose-derived MSCs in woven PCL scaffolds with fibrin and without exogenous growth factors was investigated. Collagen-rich ECM was found to completely encapsulate the fiber/hydrogel scaffolds and fill the inner pores. Whereas the fiber alone scaffolds were only encapsulated by the collagen-rich ECM after 28 days of culture.

9. Small Molecules for Directing Chondrogenesis

Current system for directing stem cell differentiation towards a chondrogenic lineage have been lacking in their ability to produce tissue comparable to articular cartilage. This tissue does contain cartilage-like ECM however lacks in the quantity produced. Therefore, other methods of directing differentiation are necessary in order to obtain comparable
tissue to the native environment. Small molecules are commonly supplemented into the culture medium for directing differentiation in vitro, including ascorbic acid and dexamethasone. However, other molecules have been explored, typically in the presence of other chondrogenic inducing molecules. Small molecules are extremely useful for in vivo applications as they can be administered locally to the site of tissue repair. Controlled release systems can also be developed to allow the small molecule delivery over time allowing for longer residence time.

9.1 Glucosamine and its analogs

Glucosamine has been studied extensively for its effects on chondrocytes. Many studies have shown that glucosamine enhances cartilage-matrix deposition along with cartilage-specific gene expression of chondrocytes. A limited number of studies have been performed to evaluate the effects of glucosamine on chondrogenesis. Glucosamine and its analogs (N-acetyl glucosamine, glucosamine oligomers from chitosan, lactose, lactosamine and N-acetyl lactosamine) have been evaluated on a chondrogenic cell line, ATDC5 cells. By evaluating alkaline phosphatase (ALP) activity, a marker for mineralization, glucosamine was found to be the only molecule capable of decreasing bone formation. Therefore, it was the only sugar evaluated in subsequent experiments. Glucosamine was shown to increase alcian blue staining for sulfate GAG and decrease gene expression for matrix gla protein (MGP), SMAD 2 and SMAD 4. MGP is a matrix protein that plays a role in mineralization of cartilage and bone. SMAD 2 is believed to regulate hypertrophy. SMAD 2 and SMAD 4 both play a role in chondrocyte differentiation. Therefore, glucosamine may have an effect on mineralization by affecting genes associated with the transition from cartilage to bone.

Hwang and colleagues looked at the effect of glucosamine on chondrogenesis of mouse embryoid bodies (derived from mouse embryonic stem cells). Glucosamine at concentrations of 2 mM and 10 mM (in the presence of TGF-β1) were shown to decrease metabolic activity and DNA content of EBs encapsulated in PEGDA hydrogels. However, at 2 mM the GAG production was significantly higher than control. Aggrecan gene
expression was also found to be enhanced at this concentration. In addition, when TGF-β1 was removed from the culture medium staining for sulfated GAG was only positive in the cells conditioned with 2 mM GlcN and the staining was comparable to groups treated with TGF-β1 alone. This is indicative that glucosamine has chondroinductive properties at a concentration of 2 mM.

9.2 NINDS library screening

The National Institute of Neurological Disorders (NINDS) has put together a library of small molecules to aid in the discovery of new therapies for neurological disorders. In it is a collection of 1040 small molecules. Huang and colleagues84 developed a system to perform high-throughput screening of chondrogenesis of bovine bone marrow-derived MSCs. Pellet culture was used as a 3D model to minimize the number of cells required to analyze a single substance. Inducers of chondrogenesis were defined as those molecules capable of increasing GAG production by a factor of 1.5 or more compared to chondrogenic medium (in the absence of exogenous growth factors). Five small molecules were found to induce chondrogenesis at concentrations of 10 μM: doxylamine succinate, pergolide mesylate, perphazine, eszopiclone and colforsin. Further investigation into each individual molecule is necessary in order to fully characterize the chondrogenic state of the cells and to optimize the concentration for induction.

10. Conclusion

Tissue engineering strategies have been developed to repair cartilage defects to ultimately reduce the prevalence of osteoarthritis. These strategies include scaffolds for directing tissue formation and stem cell differentiation in addition to small molecules for directing stem cell differentiation. This chapter has been a brief summary of articular cartilage architecture, osteoarthritis, and surgical techniques for repairing cartilage defects along with the current research areas designed to enhance the surgical techniques available.
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1. Introduction

Healthy articular cartilage is vital for proper functioning of our joints. However, the absence of neural tissue and blood vessels coupled with a sparse cell population and slow metabolic activity greatly limits the reparative process of this connective tissue. Articular cartilage can be damaged in numerous ways, including congenital pathologies such as paediatric growth plate disorders, trauma induced injuries and age related degenerative joint disorders such as osteoarthritis. Current treatment for cartilage damage has been primarily symptomatic ranging from the use of analgesics and anti-inflammatory drugs to surgical interventions that include debridement, cell therapy/grafting, joint replacement and joint fusion. These methods have had varying success with either partial healing or the formation of fibrocartilage which is mechanically inferior to native hyaline cartilage.
Functional tissue engineering offers hope to the field of cartilage regeneration/repair by combining three basic elements: (1) a suitable source of cells that have a high chondrogenic potential, are easily expandable and can be maintained for long periods of time in culture; (2) a biocompatible and biodegradable porous scaffold to act as a carrier to deliver and/or support cell growth; and (3) bioactive regulators which could be either biomolecules such as transforming growth factor-β3 (TGF-β3) or bone morphogenic proteins (BMPs) and/or mechanical factors such as hydrostatic pressure, stress or strain which can stimulate cell proliferation and differentiation.

Successful repair of cartilage defects by autologous chondrocyte transplantation was first reported in 1994 by Brittberg et al. A year later Genzyme employed this technique and produced “Carticel”, an FDA approved autologous chondrocyte implantation (ACI) treatment for patients with knee cartilage injuries. Autologous grafts or chondrocyte transplantation have been shown to be efficacious, however, they involve an invasive procedure with limited cell availability, loss of differentiation capability in vitro and a risk of donor site morbidity. Owing to their intrinsic properties, stem cells are now emerging as a favourable cell source for use in cartilage tissue regeneration. However, the application of embryonic stem cells (ESCs) is not ideal due to ethical issues and problems on the regulation of cell differentiation in vivo. For these reasons, adult mesenchymal stem cells are preferred for cell based therapies.

A stem cell is defined as a cell which possesses the ability to self-renew and differentiate into multiple mature cell lineages. Adult stem cells/multipotent cells possess limited self-renewal capabilities unlike embryonic stem cells which are considered to be pluripotent. Many researchers are investigating the trans-differentiation capacity/plasticity of adult stem cells which remains a highly controversial subject. In spite of the numerous questions regarding their trans-differentiation capabilities, mesenchymal stem cells (MSCs) have emerged as a popular cell source in cartilage tissue engineering. MSCs cultured in vitro have been documented to lack major histocompatibility complex (MHC) class II cell surface markers. These cells possess only class I MHC surface markers without any co-stimulator molecules making them an ideal source in auto/allo/xenogenic therapeutic applications. It is also well understood that MSCs have a
trophic effect, i.e. depending on their local environment and activity status they secrete large quantities of bioactive molecules which help bring about a therapeutic response.\textsuperscript{11} The presence of MSC immunomodulatory and trophic functions \textit{in vitro} has opened up a new era of cell mediated therapies. In the last five to ten years a plethora of sources of stem cells with chondrogenic potential have been reported. However, there is no uniformly accepted definitive phenotype and/or cell surface markers to assist with MSC isolation. Thus, in order to be classified as an MSC by the International Society for Cytotherapy, the cell population must: (1) adhere to plastic under standard culture conditions, (2) express cluster of differentiation (CD) markers — CD105, CD73 and CD90, and (3) be able to differentiate down chondro/osteo and adipo-genic lineages.\textsuperscript{12} This chapter will present a concise synopsis of adult stem cell sources, functions and identities (e.g. bone marrow, fat pad, synovial fluid, dental pulp, periosteum) with potential for use in cartilage tissue engineering along with a discussion on the more recent discoveries of stem cell sources.

2. Human Bone Marrow Mesenchymal Stem Cells (hBMMSCs)

An estimated one in every 10,000 nucleated cells in the bone marrow of newborns is a stem cell.\textsuperscript{13} hBMMSCs form the non-hematopoietic component of the stem cells found in the bone marrow. Friedenstein \textit{et al.} (1960s) provided the earliest evidence of the existence of stem cells in the bone marrow when they generated a haematopoietic ossicle by transplanting a whole bone marrow under the kidney capsule in mice.\textsuperscript{14} Since then, the field of mesenchymal stem cell research has gained immense popularity, with scientists regularly reporting new sources for MSCs. Bone marrow mesenchymal stem cells (BMMSCs) still remain the most studied and best understood stem cell source used in cartilage tissue engineering. Bone marrow aspirates (containing the BMMSCs) are easily accessed by introducing a needle directly into the bone marrow, mostly at the iliac crest.\textsuperscript{15,16} The ability of BMMSCs to undergo successful chondrogenesis has been studied in a variety of models. The most popular are high density cell pellets/micromasses or cell-3D scaffold constructs \textit{in vitro} and \textit{in vivo} under various mechanical conditions with/without the
addition of bioactive regulators. A typical chondroinductive cocktail contains dexamethasone (Dex) and ascorbic acid with/without a growth factor in a serum free culture medium. Positive identification of an appropriate cartilaginous phenotype is based mainly upon observed expression of the transcription factor Sox9 and the extracellular matrix proteins collagen type II, aggrecan and cartilage oligomeric matrix protein amongst others.

Numerous studies have looked into the factors that contribute to the chondrogenic differentiation of hBMMSCs. For example, chondrogenic preconditioning of hBMMSCs in aggregate cultures with fibroblast growth factor-2 (FGF-2) was observed to enhance chondrogenesis in tissue engineered constructs while withdrawal of TGF-β3 was observed to differentiate the hBMMSCs to a hypertrophic state. Enhanced glycosaminoglycans (GAG) synthesis was observed when hBMMSCs were cultured in a hyaluronan-alginate layer culture system.

Evaluation of the efficacy of hBMMSCs to undergo chondrogenesis in vivo has yielded mixed results in animal models. BMMSCs seeded on various scaffolds and implanted into rabbit cartilage defects resulted in hypertrophy of the tissue with large areas of bone replacement visible. BMMSCs seeded on a poly(lactic acid) (PLA)-alginate amalgam in a canine model led to the formation of fibrous tissue. Chen and colleagues (2005) showed the formation of cartilage-like tissue in a sheep defect model when they seeded autologous BMMSCs on a poly(lactic acid-co-glycolic acid) (PLGA) scaffold. Cartilage-like tissue formation was observed from four to eight weeks without any visible signs of bone formation. Autologous hBMMSCs seeded on a collagen gel and implanted in the patello-femoral joint covered with autologous periosteum or synovium led to an improvement of clinical symptoms with the defect being fully repaired after 12 months with formation of fibrocartilage tissue in one of the patients. Yang et al. have shown the potential of using hBMMSCs in combination with biomimetic biomaterial scaffolds to form cartilage-like tissues in different in vivo models (Fig. 1).

One of the major drawbacks observed when working with hBMMSCs is the difficulty in maintaining their plasticity in vitro. The “Hayflick limit” in hBMMSCs has been observed where successive passaging in vitro leads to a reduced proliferation and differentiation capacity.
Another drawback in the lack of a definitive *in vivo* model using hBMMSCs to generate high quality hyaline cartilage, which requires further research for the understanding of the basic biology of hBMMSCs and their behavior in a physiological condition.
3. Adipose Derived Mesenchymal Stem Cells (ASCs)

Adipose tissue (mesodermal origin) has been a recent hot bed for the discovery of stem cells. ASCs were first formally documented by Zuk and colleagues in 2001 in human lipoaspirates. ASCs are isolated from fat tissues via collagenase digestion and differential centrifugation followed by plastic adherence. The white adipose tissue (WAT) present mainly in the intra-abdominal viscera and subcutaneous tissues contains higher numbers of ASCs with enhanced proliferation and plasticity properties in comparison to brown adipose tissue (BAT) which is generally present in abundance in newborns.

Similar to hBMMSCs, ASCs have been found to be immunosuppressive due to the absence of MHC class II antigens on their surface. In addition, ASCs have been observed to exert an inhibitory effect over allogenic lymphocytes in vitro. However, freshly isolated ASCs are capable of eliciting a T-cell proliferative response at passages 0 and 1 which disappears in latter passages.

2D culture of ASCs is unfavourable for maintaining chondrogenic phenotype but culturing these cells on an elastin like polypeptide was observed to significantly enhance chondrogenesis. Using a chondroinductive cocktail including TGF-β, expression of collagen type II, aggrecan and sulphated proteoglycans was observed. Enhanced chondrogenesis of ASCs was observed when the cells were cultured with BMP-6. In contrast, hBMMSCs underwent osetogenesis upon BMP-6 exposure. Henning et al. (2007) further showed that a combination of TGF-β with BMP-6 was the ideal cocktail for chondroinduction of ASCs.

In vivo studies using ASCs have shown that these cells are capable of retaining their chondrogenic phenotype for up to 12 weeks. Fibrin glue scaffolds seeded with ASCs and implanted in vivo for eight weeks expressed aggrecan and type II collagen.

Much controversy lies in determining whether ASCs have higher chondroinductive abilities in comparison to hBMMSCs. hBMMSCs are reported to possess a higher chondrogenic potential than that of adipose tissue derived MSCs in the pellet culture system in the presence of TGF-β1/β2. However, Lee et al. (2004) reported ASCs were superior to bone marrow stromal cells in respect to maintenance of proliferating...
ability. Microarray analysis of gene expression revealed differentially expressed genes between ASC and bone marrow stromal cell. But their phenotypes and the gene expression profiles are similar.41

A drawback with the use of ASCs is their expression of embryonic markers such as Oct-4, UTF-1 and Nodal in short term culture. This has the potential to trigger the transformation of these cells into cancer-like cells over extended periods of culture.42

4. Periosteum Derived Stem/ Progenitor Cells (PDSCs/PDPCs)

The outer surface of bones at the non-articulating junctions are covered by a thin layer of connective tissue known as the periosteum. The outer fibrous layer of the periosteum is made up of fibroblasts while the inner cambial layer contains progenitor cells responsible for increasing bone width.43 The presence of progenitor cells in the periosteum that are capable of differentiating into the three mesodermal lineages (oseto/adipo/chondrogenic) has been demonstrated since the early 1990s.44 de Bari et al. (2006) isolated and characterized PDPCs with MSC surface markers. These PDPCs were observed to possess MSC-like multipotentiality when analysed via single cell lineage analysis and they underwent chondrogenesis when cultured as micromasses.45

A major plus point to using PDPCs is their ability to proliferate at a higher rate compared to BMMSCs.46 PDPCs are routinely used in the clinic to regenerate bone, however their clinical use in repairing cartilage is undocumented so far.47,48 The ability of PDPCs to undergo chondrogenesis in vivo was demonstrated in a rabbit model where the formation of ectopic cartilaginous tissue was observed 20 days following dissection of the periosteum (7 × 15 mm² periosteum defect). However, the newly formed cartilaginous tissue turned to bone by day 40. The advantage of such a technique is the elimination of the need for cell culture.49 In another report, a similar approach was used to create an artificial space (“bioreactor”) between the tibia and periosteum in which angiogenesis was inhibited (via local administration of the anti-angiogenic factor suramin in a hyaluronic acid (HA)-based gel matrix) to promote a more hypoxic
environment within the “in vivo bioreactor” space. Cartilage formation was observed within the in vivo bioreactor after ten days.50

While these cells possess MSC-like multipotentiality and higher proliferation rates than hBMMSCs, a major disadvantage of using PDSCs is the need to carry out a surgical procedure for their isolation, limiting their application as a potential autograft transplant. The probability of cell loss in the cambial layer when harvesting the periosteal cells coupled with the variation in MSC numbers in the cambial layer depending on donor age yields inconsistent results.51,52

5. Synovium Derived Mesenchymal Stem Cells (SMSCs)
The non-articular surfaces of diarthrodial joints are lined by a thin membranous tissue known as the synovium. The main function of this tissue is to maintain the synovial fluid cavity which nourishes the articulating cartilage.53 De Bari and colleagues (2001) successfully extracted MSCs from the synovial membrane for the first time.54 The immunogenicity of these cells has been found to be similar to that of hBMMSCs. SMSCs are negative for the expression of class II MHC molecules and suppress T-cell proliferation in a mixed lymphocyte reaction.55

Numerous evidences point to the chondrogenic potential of these multipotent cells. Crawford et al. (2006) reported the ability of cell cultures isolated from synovial nodules from a patient suffering from primary synovial chromatosis (PSC) to undergo chondrogenic differentiation. The benign synovial metaplasia in PSC is thought to arise due to the proliferation of mesenchymal progenitor cells.56 The expression of chondrogenic markers such Sox9, aggrecan and cartilage oligomeric matrix protein (COMP) has been documented to be 2%–25% higher in synovial calf tissue when compared to committed articular cartilage.57

SMSCs have the highest proliferation ability in the presence of TGF-β1, IGF-I and FGF-2, while synergistic action of TGF-β1 and IGF-I enhanced SMSC chondrogenesis.58 Shirasawa et al. (2006) documented enhanced human SMSCs chondrogenesis in pellet culture as a result of the synergistic effects of TGF-β3, Dex and BMP-2.59 However, further studies reported the inhibitory effect of Dex on BMP-2 chondroinduced synovial cells.17,60
In vivo, like PDSCs, SMSCs migrate from the synovium to partial thickness articular cartilage defects in adult rabbits and yucatan pigs but the cells differentiated into fibroblasts and filled the cavities with a fibrous connective tissue. Pei et al. (2009) seeded allogenic SMSCs on a polyglycolic acid (PGA) mesh, cultured the resulting constructs in vitro for one month and then implanted the neo-tissue into full thickness femoral condyle cartilage defects in rabbits. They observed the formation of hyaline-like cartilage tissue with no detectable levels of collagen type I.

Although the main advantage of using SMCs is their high proliferative capacity and enhanced chondrogenic differentiation capabilities compared to other MSCs, however, the invasive surgery needed to access these stem cells coupled with an unclear understanding of their differentiation mechanism may hinder their popular use.

6. Human Dental Pulp Stem Cells (HDPSCs)

HDPSCs have been isolated mainly from the pulp tissues of third permanent molar teeth and can be maintained for up to 25 passages. A number of studies have reported that HDPSCs could be a suitable cell source for bone tissue engineering but there are few references reporting their potential in cartilage regeneration. A STRO-1(+) DPSC population has been thought to possess a higher multilineage potential compared to non-sorted cells, probably because of their homogeneous nature. These STRO-1(+) cells can be differentiated down neurogenic, osteogenic/odontogenic, adipogenic and myogenic lineages. However, chondrogenic differentiation of these primary cells was found to be inferior to myogenic differentiation by the same cells.

Wei and colleagues (2008) demonstrated multilineage differentiation in all HDPSC samples with collagen type II expression levels being significantly upregulated after chondro-induction. The main advantage of using HDPSCs lies in the ease of obtaining these cells without any invasive surgery by banking milk teeth. However, their superiority in terms of chondrogenic differentiation over the other stem cell sources available still needs to be determined by a set of robust comparative studies.
7. Umbilical Cord/Cord Blood Derived Stem Cells

Wharton’s Jelly in the human umbilical cord has recently been reported to possess MSC-like cells (human umbilical cord perivascular cells: HUCPVCs) and depending upon how these cells are harvested, they can differentiate down the neuronal or cardiac pathways. These HUCPVCs are located close to the vasculature of the cord and have a colony forming fibroblast frequency of about 1:3000, expressing an immuno-privileged and immuno-modulatory phenotype. A comparison of the chondrogenic potential between these cells and hBMMSCs revealed that when cultured as pellets for 21 days along with TGF-β3, HUCPVCs formed larger sized pellets than HBMMSC pellets. The sGAG content and alcian blue-picrosirius staining intensities were comparable between the two cell types indicating similar matrix formation. Unlike hBMMSCs, these cells demonstrate the potential to grow in multi-layers, overlying cellular aggregates. It is however important that independent studies ascertain the possibility of these cells transforming to a cancerous phenotype and to identify the molecular mechanism involved in such multi-layered cellular growth.

Unrestricted somatic stem cells (USSCs) derived from cord blood are believed to be a promising source of therapeutic stem cells and can be differentiated down all three germ layers (e.g. ectoderm, mesoderm and endoderm). However, when compared to hBMMSCs cultured as pellets for 21 days, there was a significant decrease in sGAG content of the USSC compared to hBMMSCs pellets suggesting reduced matrix formation. Further studies of chondro-induced 3D constructs of cord blood stem cells also showed lower levels of sGAG as compared to native cartilage.

8. Other Potential Cell Sources with a Chondrogenic Potential

As an alternative to MSCs, mesenchymal progenitor cell populations (MPCs) were recently isolated from traumatised muscle tissue that had been surgically debrided due to an orthopaedic wound. The MPCs had similar morphological, proliferation and differentiation capacities as
hBMMSCs. However, before research using these cells can progress, it is important to demonstrate that they can undergo robust *in vitro* expansion and maintain their properties and differentiation capabilities in an *in vivo* environment.

The anterior cruciate ligament (ACL) has been recently identified as another source for MSCs with chondrogenic potential. Stem/stromal cells from ACL can be enzymatically released and cultured as monolayers on plastic dishes. These cells were observed to have similar phenotypic characteristics as hBMMSCs. FGF-2 and TGF-β1 can enhance the proliferation of these cells along with extracellular matrix protein production. However, in other studies, only one cell line out of six donors was found to be capable of tripotent differentiation. ACL derived cells were observed to be more beneficial in the formation of ligament fibroblasts than cartilage tissue.

The dogma of articular cartilage being a non-regenerative tissue is now increasingly being challenged with the hypothesis of the presence of a progenitor/stem cell population in the superficial layer of articular cartilage. Morphological variations are observed in the different layers of articular cartilage: cells of the flattened superficial zone secrete lubricin; the rounded and columnar arranged middle zone cells produce cartilage intermediate layer protein (CILP); while the considerably larger deep zone cells express type X collagen and alkaline phosphatase. Hayes *et al.* (2001) demonstrated the presence of slow-cycling cells, akin to progenitor cells, in the superficial zone of articular cartilage through the use of BrdU (bromo-deoxyuridine) injections. These cells expressed Notch-1 and possessed a high colony-forming efficiency.

Grogan and colleagues (2009) demonstrated the localization of progenitor cells in healthy cartilage using Notch-1, Stro-1, and vascular cell adhesion molecule-1 (VCAM-1) as stem cell markers with highest frequencies of labelled cells being observed in the superficial zone. Progenitor cells were also isolated from osteoarthritic (OA) cartilage. The frequency of Notch-1, Stro-1 and VCAM-1 positive cells was found to be higher in the middle and not the superficial zone for OA tissue. However, a similar frequency (0.14 ± 0.05%) of progenitor cells in healthy and OA cartilage was observed. These progenitor cells possessed chondrogenic and osteogenic capabilities but not adipogenic differentiation potential.
However, the authors suggested that Notch-1, Stro-1 or VCAM-1 may not be useful in identifying progenitors in cartilage and that their increased expression in OA cartilage perhaps indicates their involvement in OA. While the presence of these cell populations highlights the possibility of cartilage possessing an intrinsic regenerative capacity, further research is required to elucidate their role before they can be used in transplant studies.

9. Conclusion and Future Directions

A myriad of sources of MSCs with chondrogenic potential have been identified. Under appropriate conditions, these cells can be maintained and differentiated down the chondrogenic lineage. Some of the MSC populations are more easily isolated and differentiated than others, yet they all offer promising solutions to the field of cartilage tissue engineering. Before selecting an appropriate MSC source for cell therapy, it is important to consider whether the cell of choice can be expanded/maintained/differentiated in vitro, cultured on a scaffold and easily assessed via conventional methods of histology. At present there is a need for more robust tests to characterise cells classified as stem cells by using clongenic-lineage-specific gene marking with multipotentiality visible under in vivo conditions. Upon fully understanding the biology of the stem cells isolated we can go on to design methodologies to stimulate these cells to repair the damaged articular cartilage tissue in the body. However, the translation of research from lab to bedside could be another challenge for functional articular cartilage tissue engineering.

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1. Introduction

The intervertebral disc forms an avascular, fibrocartilaginous joint between adjacent vertebral bodies and provides flexibility while routinely supporting several multiples of body weight. The disc is composed of three major sub-tissues, the gelatinous nucleus pulposus (NP), the fibrous annulus fibrosus, and cartilaginous endplates (Fig. 1). The NP is centrally located and composed primarily of sulfated-glycosaminoglycan (GAG), type II collagen, and water. The NP serves as the osmotic mechanism that generates volume and hydrostatic pressure because the high GAG content makes the tissue very hydrophilic. The annulus fibrosus is firmly attached to the vertebral edges to serve both as a ligament to guide intervertebral movement, and as a barrier to contain nuclear swelling and thereby allow disc pressurization. The endplate is a thin (0.1 to 1.6 mm) hyaline cartilage layer that separates the NP from the adjacent vertebra. The endplate functions as a semi-permeable membrane to allow diffusive communication between disc nuclear cells and vertebral vasculature, as well as to prevent large molecular weight GAG from leaving the nuclear space.
Pain of spinal origin afflicts most adults at some point in their lives: the annual US incidence of acute and/or chronic back pain is approximately 100 million.\(^1\) Intervertebral disc degeneration underlies several painful low back disorders including intervertebral disc herniation (IVDH), degenerative spondylolisthesis (DS), spinal stenosis (SS), and degenerative disc disease (DDD). For the first three (IVDH, DS and SS), recent randomized prospective clinical studies have demonstrated advantages of surgical care compared with non-operative care.\(^1,2\) However, DDD management remains the most difficult challenge because the underlying source of pain is unclear, causing uncertainty when developing guidelines for operative and non-operative care and therapies with improved efficacy.\(^3\) As a result, estimates suggest there are between 1.5 and 4 million adults in the US with DDD-related chronic low back pain (CLBP) that have failed conservative management and await therapeutic intervention, of which there are few options beyond spinal fusion.\(^2,3\)

CLBP is a common indication for spine fusion surgery, and more recently total disc replacement. Although success rates from spinal fusion are in the range of 70\%, has several disadvantages including a decrease in spinal range of motion and acceleration of degeneration at adjacent levels.\(^4,5\) Disc replacement (where a prosthetic disc is implanted between

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**Fig. 1.** Mid-sagittal sections through a health (left) and moderately-degenerate (right) human disc. In the healthy state, the nucleus possesses a significant capacity to swell and support spinal forces. The early stages of degeneration are characterized nuclear fibrosis and annular fissuring.
vertebrae) is an attractive alternative to fusion since it maintains near-physiologic movement, but there are currently barriers to widespread use, such as limited insurance reimbursement, and potential surgical and implant-related complications.\textsuperscript{6,7} Spine fusion will likely remain the standard of care for advanced disease, where arthritic changes prevent return of normal function even with newer motion-sparing technologies. The concept of minimally-invasive biologic disc repair for less advanced cases of degeneration has grown in recent years. These include gene therapy, injection of various growth factors, and cell implantation (with or without scaffold).

2. The Demanding Intervertebral Disc Environment

The process of age-related disc degeneration can be considered chronic dysfunctional matrix remodeling in response to physical inputs such as impaired transport and/or abnormal mechanical loading, with the response to both likely modulated by yet undefined familial risk factors.\textsuperscript{8} At an early age (before ten years) there is a marked decrease in endplate vascularity and beginnings of structural disorganization. After age 20, the disc becomes sealed-off from the vertebral blood supply by the cartilage endplates and subchondral bone.\textsuperscript{9,10} Thereafter, disc cell survival is dependent on diffusion from capillaries in the adjacent vertebra (for nuclear cells) and surrounding vascularized tissues (for annular cells).\textsuperscript{11} The capillaries within the vertebra terminate just above the hyaline cartilage endplate, providing a continuous capillary bed across the bone disc interface.\textsuperscript{6} Once nutrients reach the endplate, movement of small solutes (e.g. glucose and oxygen) pass through disc matrix primarily by diffusion.\textsuperscript{11–15} (larger solutes may also be influenced by convective fluid flow created by mechanical disc compression and recovery). Cells compete for nutrition, making it difficult to sustain high cell densities at the long distances from the nutrition source typical of human lumbar discs (approximately 8 mm).\textsuperscript{12–15}

This disc transport limitation has several negative consequences. Tissue oxygen concentrations are low, in the range of 0.5% to 5%.\textsuperscript{16} These hypoxic conditions inhibit matrix synthesis: sulfate incorporation at 1% oxygen is one-fifth that at 5%.\textsuperscript{11,16} Because of limited oxygen, the nucleus pulposus cells produce energy through anaerobic glycolysis, which utilizes glucose and generates lactic acid as a by-product.\textsuperscript{11,14} Accumulation of
lactic acid decreases disc pH (to near pH 6.3) and is detrimental to the matrix as it decreases glycosaminoglycan production, tissue inhibitor of metalloproteinases (TIMP) production, and cell viability. The dependence on anaerobic glycolysis for cell production of ATP makes glucose a critical nutrient. Disc glucose concentrations are typically considered to be in the range of 0.5 to 5 mM, where disc cells die within 24 hours at concentrations below 0.2 mM. Other factors in serum are also important as serum deprivation results in decreased cell proliferation and increased cell senescence. Moreover, the influence of these factors is not necessarily independent, since some research indicates hypoxia supports nucleus cell survival during serum withdrawal.

Another main feature of the disc nucleus is osmolality. The disc functions biomechanically by using a high osmotic pressure (generated by proteoglycan fixed charge) to attract water and produce physical pressure to support spinal compression. This osmotic stress (in the range of 250 to 450 mOsm) causes changes in cell volume and stimulates cell behavior via cytoskeleton rearrangement. Under hypo-osmotic conditions (250 mOsm) disc cells increase gene expression for aggrecan and type II collagen, while at hyperosmotic conditions (450 mOsm) there is a downregulation of biglycan, decorin and lumican in nucleus pulposus cells.

3. Evaluating a Stem Cell-Based Therapy

3.1 In vitro outcome measures and assessments

Since disc degeneration is considered to initiate in the nucleus, regenerative strategies target nucleus regeneration. As a result, stem cell-based therapies focus their efforts on replicating the characteristics of the native nucleus pulposus cells (NPCs). Stem cell performance is typically evaluated by characterizing gene expression and the matrix synthesis. In order to assess differentiation stage and the cell fate, the gene expression levels are measured for the positive chondrogenic marker Sox9, as well as several negative markers including the fibroblastic marker Collagen 1, the hypertrophic marker Collagen X, and the osteogenic marker Runx2. Major matrix proteins aggrecan and Collagen 2 are often evaluated to assess the cells' level of protein synthesis. In addition, matrix metalloproteinase (MMP) genes that are responsible for degrading tissue are generally measured, in
particular MMP-2, MMP-9 and MMP-13 are commonly measured in degenerate discs.\textsuperscript{19} For quantitative analysis at the protein level, proteoglycans are typically measured using a dimethylmethylene blue assay and Collagen 2 can be quantified using an enzyme-linked-immunosorbent-assay.\textsuperscript{20,21} Both proteins can also be assessed qualitatively using histologic staining with Safranin-O or immunohistochemistry techniques.\textsuperscript{20}

Biological efficacy should be demonstrated in models of increasing complexity. Two- and three-dimensional cell culture systems can be useful for initially demonstrating cellular effects, dosing, and toxicity. Three-dimensional systems are preferable to maintain cell phenotype, and augmenting stimuli to include other disc mimetic conditions such as pressure, hypoxia, and inflammation are important as these factors can significantly influence cell function.\textsuperscript{22} The degenerative disc conditions will vary with the degree of degeneration, however, the cells implanted in a human degenerative disc will generally experience hypoxia (about 4% O\textsubscript{2}), high pressure (350 KPa at rest), inflammatory cytokines (particularly TNF-alpha and Il-2b), and a drop in pH (as low as pH 6.7). To simulate those conditions, a number of different methods can be used. The oxygen content can be controlled with a hypoxic incubator and a pressurized environment can be stimulated with the aid of a bioreactor. In addition, the inflammatory cytokines and pH levels can be replicated in the culture media. Therefore, the \textit{in vivo} degenerative disc environment can be mimicked \textit{in vitro} and should be considered during testing and optimization.

\subsection*{3.2 Models for efficacy and safety: \textit{In vivo} preclinical models}

Ultimately small animal studies are a critical next step because of the important \textit{in situ} interactions between disc cells and spatially-varying host features unique to the healing disc environment: pressure, hypoxia, degraded matrix, cytokines, other stromal and inflammatory cells, plus systemic factors. Along with an increasingly complex model come challenges in response interpretation. Outcome measures should be coupled to the designed treatment mechanisms, but should also include overall indices of disc quality such as histology and biomechanics. Time dependence of the outcomes is critical to establish whether the therapeutic response is persistent above the background degenerative response typically triggered by the therapy delivery. Design of experiments (DOE)
techniques for study design and statistical analyses can help establish sample size and efficiently optimize treatment parameters. Safety also needs to be established in preclinical models. The avascular nature of the disc environment can lead to persistence of active therapeutic agents, secreted cytokines, as well as carrier degradation products. Consequently, even though a growth factor or carrier has an established use track record in other tissues, they need to be evaluated in the unique NP environment. Adverse reactions can manifest through inter-discal toxicity, inflammatory cell recruitment, and matrix erosion. For example, cytokines and scaffold degradation products can diffuse from the disc and incite a sclerotic reaction in the adjacent vertebral endplates, along the delivery wound site, or outside the annulus fibrosus. Equally important is to establish the reaction to extradiscal placement of the therapeutic materials and delivery vehicles. It is likely that these can escape from the disc during surgery or early in the post-surgical period. Inflammation and the mass effect induced by these materials can adversely affect adjacent nerve roots and other paraspinal tissues. The safety of injected materials or cells should also be confirmed under the worst case scenario to avoid a catastrophic event, such as the one seen for chemonucleolysis.

After efficacy and safety are established in vitro in small animals, large animal studies are required to motivate clinical use, principally because of size effects on disc transport and biomechanics. Typical animals used for this purpose include goats, sheep and mini-pigs. As with other preclinical models, efficacy may be difficult to establish due to a lack of relevant starting points and clinical metrics that match the intended patient population. Yet, biologic plausibility should be supported as well as safety through histological, biochemical, and biomechanical assays. Comparisons to negative controls (surgical procedure without treatment delivery) and untreated levels can help judge effect size and potential clinical relevance.

4. Non-Stem Cell-Based Regeneration Strategies

4.1 Gene therapy and growth factors

Several groups have published promising results using either in vivo or in vitro gene therapy. Growth factors such as TGF-beta, BMP2;
transcription factor Sox9; inhibitors IL1Ra (interleukin-1 receptor antagonist) and TIMPs have been successfully delivered to NPCs. Another approach that modulates cells at the gene level is RNA interference which is designed to silence genes that are the potential cause of disc degeneration without needing a viral delivery vehicle.28

The most straightforward acellular biologic strategy is to inject growth factors into the disc. Several studies have reported in vivo effects of OP-1 (osteogenic protein-1), TGF-β (transforming growth factor-beta), GDF-5 (growth differentiation factor-5) that have led to an increase in disc height and GAG content.29 Although promising results have been reported using these techniques, the relative acellularity of human degenerated discs raises the concern that the patient’s own disc cells may be insufficient to mount a therapeutic response. Other concerns regarding these strategies include the activation of the host immune response due to the presence of viral vectors during gene therapy and the short half-life of bioactive molecules used in growth factor therapy.

4.2 Autologous NPCs

The introduction of cells capable of surviving within the intervertebral disc and producing appropriate amounts of matrix is an important component of disc tissue engineering. One type of cell considered is an autologous (derived from the patient) NPC cell-line.30 Even with some promising data, there is legitimate clinical concern over donor-site morbidity, since harvesting the patient’s own cells requires damage to an adjacent disc, which will likely induce degeneration in that level. Also, disc acellularity will require a slow in vitro cell-culture expansion step to obtain sufficient cell numbers. Furthermore, autologous cells will be similarly aged to the diseased level and potentially limited in their ability to mount therapeutic repair response.

5. Stem Cells for Disc Repair

5.1 Cell carriers

Carriers for cell-based disc regeneration strategies fulfill multiple roles. They serve as delivery vehicles to attain acute cell retention in the high-pressure
disc nucleus. They preserve nucleus volume and defend against scar tissue encroachment by adjacent annular tissue during early healing. By augmenting nuclear volume, carriers also serve to enhance acute biomechanical stability. In addition to these biomechanical functions, carriers need to provide an environment that supports the desired biological activity of the delivered cells. Unfortunately, biomechanical and biological roles may create conflicting design constraints, with stiffer materials being more suitable for biomechanical retention and stability, and porous, pliant materials being more appropriate for nutrient transport and a 3D milieu conducive to a disc cell phenotype. Importantly, these materials, which may or may not degrade over time, have to be synergistic with cell function over the long term. If non-degrading, they need to be biocompatible and non-migratory under complex loading/pressures. If degrading, the degradation kinetics should ideally be timed with cell matrix synthesis. Also, because of disc size and avascularity, degradation products may have a longer persistence and achieve higher concentrations than observed in other applications.

Many types of carriers have been used that include gels with or without porous solid scaffolds. Examples include synthetic polymers such as poly(lactide-co-glycolide) (PLGA), polyglycolide (PGA), and polylactide (PLA). Many forms of natural scaffolds are available, such as hyaluronan, collagen/atalocollagen, chitosan, alginate, agarose, calcium polyphosphate, demineralized bone particles, fibrin sealant, and small intestine submucosa (SIS) — a natural extracellular matrix. So-called smart scaffolds can contain bioactive agents such as growth factors, cytokine inhibitors, or antibiotics.

5.2 Autologous versus allogenic

Adult mesenchymal stem cells (MSCs) are attractive for disc tissue engineering since they can differentiate into a variety of cell types, including NPC-like cells. Depending on the therapy, the MSCs can be autologous or allogenic (derived from a donor). In the case of autologous transplantation, the patient would have a preliminary outpatient procedure where MSCs would be harvested from bone marrow or adipose tissue. Since MSCs represent only a small percentage of the cells in either of these
donor tissues, the MSCs would need to be separated and expanded \textit{in vitro} to have sufficient numbers desired for the therapy. The implantation procedure would be performed several weeks later. In the case of an allogenic transplant, the patient would be treated with MSCs from an organ donor in a one-procedure cost-effective approach. While host rejection of allogenic cells is a concern, several studies have indicated that MSCs are immunoprivileged and do not elicit a rejection response.

5.3 \textit{Differentiation of stem cells before implantation}

A primary concern regarding the use of MSCs for disc repair is whether they survive the harsh \textit{in vivo} conditions and appropriately differentiate \textit{in situ}. Although the NPC lineage is not fully characterized, it is generally agreed to closely match that of chondrocytes.\textsuperscript{43,44} While MSCs are known to readily differentiate into this cell type under controlled conditions \textit{in vitro}, it must still be established whether they will spontaneously differentiate and thrive \textit{in situ}, or alternatively require augmentation with supplemental, differentiation factors.

Environmental cues can provide MSCs with important differentiation signals. Several studies have traced labeled MSCs implanted within discs and observed that they persist, integrate with host tissue, and differentiate over time.\textsuperscript{45,46} Similarly, \textit{in vitro} studies have shown that MSCs cultured in 3D scaffolds also exhibit some levels of differentiation.\textsuperscript{47,48} Yet, several studies have shown greater persistence and matrix deposition with MSCs that are first predifferentiated or implanted along with stimulatory factors.\textsuperscript{49} Consequently, the introduction of key bioactive molecules is commonly used to enhance desired MSC differentiation.

The use of adenoviral vectors encoding chondrogenic growth factors in MSCs is one possible strategy to ensure the differentiation and sustained performance of implanted MSCs. Gene transfer therapy enables the sustained synthesis of the encoded bioactive transgene products that may be effective since these factors would not likely be present in a degenerative \textit{in vivo} environment. The transfer of key genes including TGF-$\beta$1 and BMP-2, to MSCs \textit{in vitro} have lead to the sustained upregulation of key matrix production and differentiation genes.\textsuperscript{50,51} Transfected cells can be implanted alone or in combination with untransfected cells.\textsuperscript{52} However,
the use of viral vectors and genetically modified cells represent important safety hurdles when considered for clinical application. These safety concerns will likely slow clinical adoption.

Exposing MSCs to key growth factors either before or during implantation is a more popular strategy. The most common approach is to culture MSCs in 3D alginate bead culture with TGF-β1 supplemented media, where MSCs have robust differentiation and matrix synthesis. This method is also convenient because the cells can easily be released from alginate using sodium citrate washes and reimplanted without compromising cell viability. Controlled release of growth factors such as TGF-β1 and other such molecules can also be incorporated into scaffolds that are seeded with cells and implanted. The main concern regarding TGF-β-induced differentiation of MSCs is the progression of MSCs towards hypertrophy whereby the MSCs begin secreting collagen X and MMP-13. This is a concern since the mechanical properties of matrix secreted by hypertrophic cells do not match those desired for disc regeneration.

5.4 Co-culture techniques

A newly emerging technique is to co-culture MSCs with mature instructive cells. This approach was originally explored to identify interactions between implanted stem cells and host cells. However, when synergistic effects were observed, co-culture was investigated for potential therapeutic benefits. In this context, co-culture involves creating 3D cell pellets that allow for contact between the two cell types. This synergy has been shown to increase overall matrix production and promote differentiation of MSC without leading to hypertrophy. The NPCs are thought to provide sustainable signaling cues to the MSCs and the MSCs are also thought to be providing the NPCs with stimulatory signals. The combination of these two effects is very attractive therapeutically in creating a self-sustaining implant that does not require external cues.

Yamamoto et al. report that cell-cell contact between MSCs and NPCs had synergistic effects in monolayer, however, it remained unclear whether the MSCs were differentiating or acting as feeder cells to reactivate the NPC. Ultimately, Richardson et al. employed a similar two-dimensional co-culture system and demonstrated that NPCs cause
MSCs to differentiate into an NP-like phenotype as assessed by gene expression after FACS sorting. They observed that a 75% NPC/25% MSC ratio was optimum for MSC differentiation, as indicated by SOX9, collagen 2, and aggrecan gene expression. Another study has since made similar observations in 3D culture using a randomized mixture of MSCs and degenerative NPCs. These studies highlight the beneficial effects of recreating a condensation shape and the unique signaling arising from coculturing. However, none have reproduced the key induction process where two layers of different cell types communicate both with heterogeneous signaling across the interface and homogeneous signaling within the layer of the same cell type.

Our current strategy seeks to regenerate the disc using a novel bilaminar cell pellet (BCP) (Fig. 2). The BCP is a co-culture pellet composed of an inner sphere of MSC enclosed in an outer shell of NPC. The cell composition ratio is 75% MSC and 25% NPC with a total of 500,000 cells and roughly a 1mm diameter. The bilaminar structure allows for homotypic interactions between cells of the same type within the layer and for heterotypic interactions between different cell types across a defined interface. This organization mimics the processes of condensation, where cell aggregates form, and induction, where a mature layer of tissue directs the differentiation of a naïve one. The two cell types provide one another with stimulatory signaling which eliminates the need for growth factors or genetic manipulation since the BCP provides self-sustaining cues.

Synergistic interactions are apparent within the BCP suggesting NPCs direct MSC differentiation. After three weeks in culture, MSCs in the BCP

![Fig. 2.](image-url) (A) Co-culture in monolayer by Yamamoto and Richardson. (B) Random 3D co-culture pellet. (C) Bilaminar co-culture pellet (BCP). (D) Frozen section histology of BCP with MSCs dyed with Dil (red).
exhibit significantly higher gene expression of aggrecan (two-fold), collagen II (675-fold), and SOX9 (175-fold) and a significant downregulation of MMP13 (three-fold) and ColX (eight-fold) over MSC controls.\textsuperscript{20} In conjunction, NPCs in the BCP exhibit significantly lower levels of expression of aggrecan and collagen II (both two-fold) but a similar level of SOX9. Spatial and temporal gene expression patterns also provide clues to the nature of cellular interactions within the BCP. At early culture times (one week), the expression of both the aggrecan and collagen II is primarily on the BCP periphery, where the NPCs are located. As time progresses, there is increased aggrecan gene expression by MSCs at the BCP center.\textsuperscript{20} Taken together, these results indicate that MSCs are differentiating due to their interaction with the NPCs within the BCP.

BCP culture results in a 30\% increase in proteoglycan production after three weeks as compared to single cell type controls.\textsuperscript{20} Consistent with gene expression patterns, early aggrecan production is primarily restricted to the outside layer presumably by the NPCs. By three weeks of culture, the aggrecan staining is widespread, indicating that the MSCs have begun synthesizing the protein (Fig. 3).

Importantly, BCPs demonstrate superior performance when cultured under conditions that mimic those anticipated for the degenerate disc environment: hypoxia (4\% $O_2$), pressure (350 KPa), and inflammation (10 ng/ml

![Fig. 3. Immunohistochemistry staining for aggrecan (dark gray) on paraffin sections of BCP. (A) BCP at one-week time point. The aggrecan staining is localized to the outer layer where the NPCs are present. (B) BCP at three-week time point. The aggrecan staining is throughout the pellet including the center of the pellet where the MSCs are located. This indicates a progression in MSC differentiation towards an NPC phenotype between one and three weeks.](image-url)
of TNF-α and interleukin 1-β). As expected, single-cell type pellets consisting of NPCs produce more matrix than MSCs alone or BCPs under physiologic disc conditions of hypoxia and pressure. However, when cultured in the presence of cytokines, BCPs and MSCs produce significantly more proteoglycan than NPCs alone. In this setting, the NPC performance was dramatically reduced indicating their high sensitivity to the inflammatory environment. When hypoxia, pressure, and inflammation are combined to simulate the pathologic disc environment, BCPs produce significantly more proteoglycan than MSCs and NPCs. These results demonstrate the sensitivity of cell performance to culture conditions, highlighting the importance of mimicking the anticipated in situ environment during in vitro optimization of cell-based tissue engineering strategies.

The resilience of BCPs to the simulated pathologic disc environment in vitro suggests advantages for in vivo application. This is borne out by preliminary studies in rat caudal discs. Two weeks after implantation using a fibrin carrier into denucleated discs, cell retention and survival was increased by 50% with BCPs versus MSCs or NPCs alone. At five weeks, the BCP-treated discs demonstrated significantly better disc-morphology (assessed histologically by a blinded-scoring-scheme) than either untreated or fibrin-only groups, and tended toward better scores than MSC- and NPC-only conditions. The BCP-treated discs uniquely exhibit histologic evidence of proteoglycan synthesis, and tended to better maintain disc height than the other groups (Fig. 4).

![Fig. 4](image)

Fig. 4. Rat disc paraffin section histology with Safranin-O staining five weeks after surgery. (A) This disc was a control disc with no treatment. (B) This disc was injected with the fibrin carrier alone. In images (A) and (B), the disc has collapsed and there is no proteoglycan in the disc space. The end plate and growth plate are severely disrupted. (C) This disc was treated with a BCP and fibrin carrier. The disc height is maintained with some proteoglycan staining in the disc space. The end plate and growth plate are both continuous.
These BCP studies indicate the value of adapting inductive strategies utilized during normal joint development to guide appropriate MSC differentiation in the challenging wound healing environment. As opposed to using a single growth factor supplement or genetic manipulation, this method leverages the totality of NPC signaling to program appropriate response in MSCs. This programming inhibits hypertrophy and promotes resistance to inflammation. BCPs synthesize substantially more disc-like matrix than either NPCs or MSCs alone. Thus, BCPs, are a promising stem cell-based approach for disc repair.

6. Conclusion

The successful design of cell-based treatments for low back pain is confounded by ambiguities of disease and pain mechanisms in patients, and lack of consensus regarding ideal preclinical models. In particular, the primary clinical endpoint — pain relief — is currently not directly testable in animals. Yet, these therapies can be advanced by establishing biologic plausibility of efficacy and safety using models of increasing complexity, starting with cell culture, small animals (rats and rabbits), then large animals (goat and mini-pig) that more closely mimic nutritional, biomechanical, and surgical realities of human application. Ultimately, success will hinge on carefully designed clinical trials with well-defined patient selection criteria and objective outcome metrics that demonstrate significant benefits relative to gold-standard control treatments, such as spinal fusion.

References


1. Introduction

The breadth of conditions afflicting the skeletal system, as well as variety of patients beset with defects and deficits of the skeletal system, is expansive. To this end, the extent of this clinical need has garnered a significant amount of effort from surgeons and researchers to develop novel therapeutic interventions aimed at achieving the end goal of improved patient outcomes. However, despite progress in surgical techniques and our understanding of skeletal biology that guides the endogenous regenerative process, reconstructive modalities currently at the disposal of surgeons continue to frequently produce less than ideal results. This largely stems from the inability of modern reconstructive techniques to simultaneously restore both the form and function of native bone. As such, the field has been witness to a shift in investigative focus in recent years. Skeletal regenerative medicine, and more specifically progenitor cell-based tissue engineering, offer a new paradigm in the treatment of disease processes involving the
skeletal system currently intervened upon utilizing standard therapies. Where current reconstructive modalities fail, ideal cell-based skeletal tissue engineering strategies are focusing on the utilization of autogenous osteoprogenitor cells paired with novel biomimetic scaffold materials capable of delivering targeted pro-osteogenic molecular signals in a spatiotemporally controlled fashion in a single operative procedure (Fig. 1). Substantial progress has been appreciated from efforts to achieve this overarching goal. However, with every novel discovery, additional light is shed not only on the critical importance of each of these components comprising a cell-based skeletal reconstructive strategy, but the need to understand the harmonious interplay that must exist between each of these components in order to result in a functionally competent skeletal regenerate.

What are the ideal osteoprogenitor cells, scaffold materials, and pro-osteogenic molecular signaling pathways that will be critical to the translation of tissue engineered bone to the clinical arena? For investigators focusing their efforts in the field of skeletal tissue engineering, answering these questions, as well as defining the interactions that transpire between each of these components during the formation of

Fig. 1. Bedside tissue engineering.
de novo tissue engineered bone, has taken center stage. Through multidisciplinary collaboration between surgeons and scientists in the fields of stem cell biology, developmental biology, and bioengineering, significant advances have been appreciated. Of note, recent advances in the study of candidate osteoprogenitor cells have moved us closer to identifying a multipotent cell population possessing the necessary characteristics for successful application in skeletal tissue engineering strategies. Additionally, work in our laboratory and others continues to advance our understanding of molecular signaling pathways critical to osteogenesis and amenable to targeted manipulations, with the goal of enhancing the osteogenic differentiation of progenitor cells. Indeed, encouraging results from in vitro and in vivo studies, as well as early human applications, have been appreciated to date.

As alluded to above, improved patient outcomes resulting from surgical intervention have been observed as a direct result of increasing knowledge regarding the molecular underpinnings guiding skeletal development and regeneration, in addition to technological advancements in skeletal reconstruction. Largely, this knowledge has been derived from the study of endogenous bone tissue engineering in the form of distraction osteogenesis. By refining our understanding of how biomechanical forces alter skeletal remodeling and regeneration, surgeons have developed and optimized techniques to harness the endogenous regenerative capacity of bone. Furthermore, the study of congenital skeletal anomalies resulting from miscues in genetic and molecular biology during the developmental process, including craniosynostosis, have yielded a wealth of information regarding signaling pathways critical to the process of osteogenesis. It is such knowledge upon which the foundation of our understanding of skeletal regenerative biology has been built and allowed the pursuit of cell-based skeletal tissue engineering strategies to evolve.

The extent of diseases affecting the skeletal system that potentially require surgical intervention impart an underappreciated yet substantial burden on the US healthcare system. Upon examination of data collected in conjunction with the US Health Care Utilizations Project, one can begin to grasp the significance of this trend. Data from 2006 regarding the
cumulative cost of intervention to treat fractures of the hip, extremities, and craniofacial skeleton surpassed US$9 billion dollars in the US.\textsuperscript{2} This did not include the treatment of pathologic fractures, upon which an additional US$1 billion dollars in expense was incurred.\textsuperscript{2} Furthermore, recent trends suggest that this burden can be expected to substantially expand in the not-so-distant future. A greater than two-fold increase in the annual number of inpatients discharged with the diagnosis of osteoporosis and other chronic diseases afflicting the skeletal system has been observed over the course of the past decade.\textsuperscript{2} Together, these data allude to the presence of a substantial and growing clinical need for novel skeletal reconstructive strategies.

In addition to escalating demand, the shortcomings of modern reconstructive interventions to treat skeletal defects and deficits, whether arising from congenital malformations, oncologic resection, or traumatic tissue damage/loss, has further heightened awareness regarding the need for disruptive innovations in the field. Despite substantial incremental improvements in modern complex microsurgical techniques and reconstructive material, current reconstructive efforts centered on autogenous tissue transfer, allogeneic tissue implantation, and the implementation of improved alloplastic materials all have shortcomings. The limited amount of autogenous donor bone that can be harvested from any of a variety of locations (calvarium, ribs, iliac crest, etc.) prior to imparting clinically significant donor site morbidity related to the harvest remains an inherent limitation of the technique.\textsuperscript{3} In addition, complications associated with allogeneic tissue and alloplastic material reconstruction include infection, immunologic rejection, and structural failure. Such deficiencies point to a clear need to improve upon our current capacity to treat skeletal disease requiring surgical intervention. In the following discussion, we will probe the historical work in which much of our current understanding of skeletal biology is founded. Subsequently, a review of the current state of studies investigating each of the critical components of cell-based skeletal tissue engineering strategies (progenitor cells, molecular biology of skeletal regeneration, scaffolds) will be presented. Finally, effort will be made to present what critical steps remain in order for the successful translation of such strategies to the clinical arena to be appreciated.
2. Lessons Learned from Endogenous Skeletal Tissue Development, Healing and Regeneration

2.1 Distraction osteogenesis: endogenous skeletal tissue engineering

At its essence, distraction osteogenesis utilizes controlled mechanical force to stimulate and guide osseous regeneration. As such, it can be viewed as skeletal tissue engineering in its purest form. The characteristics of bone formation observed following successful distraction are representative of those desired from a cell-based skeletal regenerate; bony healing over the course of a relatively short period of time that results in a regenerate of similar in size, shape and quality as the native bone. First described by Codivilla in 1905 using a limb model, several decades passed prior to Gavril Ilizarov describing the physiologic and mechanical factors necessary in governing successful regenerate formation using a long bone fracture model.4,5 Subsequently, similar principles were applied to the craniofacial skeleton. First studied using animal models in the 1970s, it was not until 1989 when McCarthy and colleagues utilized distraction in the human craniofacial skeleton to lengthen the mandible.6 Subsequently, distraction osteogenesis has grown to be the cornerstone of surgical intervention to treat a multiplicity of mandibular and midface deficiencies.7

Over the course of distraction osteogenesis, deficient bone is initially osteotomized. Subsequently, following a period of latency, the osteogenic fronts are gradually separated from one another.7 A period of consolidation follows, during which callus formation and de novo bone formation are observed. Additionally, during the course of osteogenic distraction, surrounding soft tissues are also observed to expand and adapt to increased regional needs.7 Thus, resulting from a complex interaction between externally applied mechanical forces and mesenchymal tissues, progenitor cells and osteoblasts are stimulated and guided to endogenously produce a skeletal regenerate with structural form and biologic function that mirrors that of native tissues. The importance of understanding the molecular mechanisms underlying this impressive endogenous skeletal regenerative capacity has not been underappreciated. Indeed, a great deal of knowledge that is
currently guiding cell-based regenerative efforts stems from lessons learned here.

For tissue engineers, harnessing an understanding of the events transpiring on a molecular level to guide the differentiation of progenitor cells toward the osteogenic lineage in the formation of mature skeletal tissue has been of utmost importance. Studies have revealed a multitude of small molecules that are elaborated during distraction osteogenesis. Of these, TGF-β, BMP, and FGF family members have emerged as important mediators of the osseous regenerative process.8–11 Furthermore, additional work has highlighted the importance of signaling mediated by FAK, MAPK/ERK, VEGF, HIF-1, and SDF-1 in guiding the processes of osteogenesis and angiogenesis critical to skeletal regeneration resulting from distraction.12–16

To elaborate, the role of TGF-β1 promoting collagen production during bone healing, as well as the inhibition of matrix metalloproteinases and osteoclasts, has been well described.17,18 Additionally, TGF-β1 may play an important role in the neovascularization of new tissue through the up regulation of the pro-angiogenic factors VEGF and bFGF.19–22 Finally, of potential interest to skeletal tissue engineers are the temporal differences in expression of TGF-β1 during distraction osteogenesis compared to fracture repair.23,24 Distraction osteogenesis is marked by an early rise and sustained expression of TGF-β1, whereas an early downregulation and subsequent late increase in expression are observed during the process of fracture healing.

Effort has also been invested in defining the molecular mechanisms that regulate the transduction of externally applied forces to stimulate de novo osseous regeneration. Bradley and colleagues probed this question by developing a system that allowed for the application of both linear distraction and compression to MC3T3 preosteoblasts suspended in collagen type I gel.25 Here, it was observed that constant distraction augmented the proliferative capacity treated cells, while cyclic distraction and compression lead to increased expression of markers of osteogenic differentiation. In vivo work by Tong and colleagues made effort to clarify what molecular signaling pathways may be contributing to these observations. Here, focal adhesion kinase (FAK) was immunolocalized to tissues comprised of regenerated bone following distraction, speaking to the important role this signaling pathway may be contributing to the process.13
2.2 Craniosynostosis

Where the study of distraction osteogenesis has lead to important observations regarding the molecular underpinnings of endogenous skeletal tissue regeneration, so to has the study of congenital anomalies of the craniofacial skeleton. Indeed, work aimed at understanding the genetic and molecular signals involved when developmental cues fail, resulting in craniosynostosis, have further matured our understanding of important biologic principles that are today important to efforts in skeletal tissue engineering.

The identification of molecular miscues resulting in craniosynostosis have contributed to our current understanding of the molecular underpinnings of osteogenic biology. Numerous signaling pathways have been implicated in the evolution of craniosynostosis, including TGF-β and FGF.26 Of particular interest is the role that bone morphogenetic protein (BMP) family members, as well as their antagonists, have been observed to play in the development of aberrant cranial suture phenotypes.27–29 To elaborate here, work by Warren and colleagues made interesting observations regarding the contribution of the BMP antagonist Noggin in normal mouse cranial suture fate.27 In their study, they were able to demonstrate that Noggin expression was observed in patent (sagittal and coronal), but not fusing (posterofrontal), sutures. However, when misexpressed via viral-delivery to the posterofrontal suture, pathologic patency was observed. Furthermore, Noggin was shown to be suppressed by FGF-2 and syndromic gain of function FGFR signaling. Thus, it can be postulated that syndromic FGFR-mediated craniosynostoses may result from inappropriate downregulation of Noggin expression. Of interest to current skeletal tissue engineering efforts, conceptually such observations speak to the potential benefits from simultaneous stimulation of pro-osteogenic agonists and inhibition of antagonists in an effort to maximize the osteogenic potential of multipotent progenitor cells.

3. Progenitor Cell-Based Skeletal Tissue Engineering

Indeed, it is the unique endogenous capacity of skeletal tissue to self-renew and regenerate subsequent to injury that makes it such an attractive area of focus for cell-based tissue engineering efforts. However, despite
this profound endogenous regenerative capacity, skeletal defects and deficits resulting from congenital anomalies, chronic disease (osteoporosis), surgical resection (oncologic), and traumatic injury frequently exceed this capacity and necessitate surgical intervention. It is here that skeletal tissue engineering and regenerative medicine aim to improve on the inadequacies of current reconstructive modalities. Despite advances in surgical technique and biomaterial technologies, less than desirable patient outcomes following skeletal reconstructive efforts are far too often observed. Thus, a shift in focus has transpired toward investigating the capacity of tissue engineering, and specifically progenitor cell-based tissue engineering, to meet this need.

What are the critical characteristics of a multipotent osteoprogenitor cell that can potentially fulfill this need? First, it is important that they demonstrate a potential to undergo efficient and predictable osteogenic differentiation. Additionally, the cells must display a robust proliferative capacity, be accessible in sufficient numbers (ideally in quantities that avoid the need for ex vivo expansion prior to utilization), and be acquired by means that do not impart undo donor morbidity.

One needs to search no further than human embryonic stem cells (hESC) to identify a cell population that clearly fulfills all of these criteria. Furthermore, utilizing a true pluripotent cell population, capable of regenerating multiple tissue types arising from all three germ layers, opens doors to the possibility of addressing increasingly complex reconstructive scenarios. However, enthusiasm surrounding the application of hESC in regenerative medicine strategies has largely been squelched by political and ethical concerns regarding their clinical application. It is these hurdles that have lead scientists to escalate investigations in pursuit of identifying a postnatal progenitor cell populations capable of displaying the aforementioned biologic characteristics necessary for application in tissue engineering strategies. Encouragingly, coming forth from work studying mesenchymal stem cells (MSC), a body of evidence has accumulated that supports their capacity to potentially meet this need. Now isolated from a multitude of adult tissues, and observed to have a multipotent capacity to differentiate down osteogenic, cartilaginous, adipogenic and myogenic lineages, efforts to expand our understanding of this cell population to meet the needs of skeletal tissue engineers has expanded.
3.1 Bone marrow-derived mesenchymal stromal cells

Earliest investigations on MSC focused on those derived from bone marrow, coined bone marrow mesenchymal stem cells (BMSC). With regard to the multipotent potential of this progenitor population, early in vitro work by Pittenger and colleagues described their ability to undergo multilineage differentiation, including osteogenic, when cultured in the appropriate milieu of small molecules.\textsuperscript{31} Since that time, a wealth of information has accumulated displaying the capacity of both BMSC harvested from small animals, as well as bone marrow aspirates derived from humans, to augment osseous regeneration \textit{in vivo}.\textsuperscript{32,33}

Despite these encouraging results, multiple hurdles have been put forth imped ing the widespread clinical application of these cells in skeletal tissue engineering strategies. Factors including donor morbidity resulting from bone marrow aspiration, as well as potential age-dependent decline in proliferative and osteogenic capacity that may exist, hinder potential for clinical application.\textsuperscript{34} Furthermore, the paucity of BMSC found in bone marrow aspirates (one in 27,000 cells) would likely necessitate their \textit{ex vivo} expansion, on the order of weeks, to obtain adequate cell numbers to produce a single reconstructive construct.\textsuperscript{35} As such, subsequent efforts on the part of investigators have focused on a more readily obtainable and abundant source of MSC to mitigate the aforementioned factors that have impeded the use of BMSC in the past.

3.2 Adipose-derived mesenchymal stromal cells

To this end, an abundance of evidence now exists that suggests that adipose-derived stromal cells (ASC) may be up to the task. Early on, Zuk \textit{et al.} observed ASC to have a similar potential for mesenchymal lineage specific differentiation as BMSC (bone, muscle, adipose, cartilage), as well as display a surface antigen profile that equated that found in BMSC with little exception.\textsuperscript{36,37} Furthermore, it appears as though ASC do not display a decline in proliferative capacity with age as was previously observed in BMSC.\textsuperscript{38} Given these early observations, paired with the beneficial characteristics of ASC compared BMSC (relative abundance and potential for acquisition with relatively little donor morbidity), our
laboratory and others have invested significant effort in pursuing the translational potential of this mesenchymal progenitor population in the setting of skeletal tissue engineering.

*In vivo* studies have put forth promising results regarding the potential to utilize ASC in the development of cell-based skeletal tissue engineering strategies. In this context, the bone forming capacity of ASC was first described by Lee and colleagues, when they observed ossification of ASC seeded PLGA scaffolds implanted subcutaneously.\(^{39}\) Subsequently, Cowan *et al.* demonstrated the ability ASC to regenerate bone in critical-sized calvarial defects.\(^{40}\) In their study, murine ASC were seeded on apatite-coated, PLGA scaffolds. After implantation into critical size parietal bone calvarial defects, comparable bony healing was noted in defects treated with ASC relative to bone formation in study groups treated with BMSC and osteoblasts.\(^{40}\) Most recently, Yoon *et al.* reported a significant increase in the healing of calvarial defects created in nude mice, and repaired with human ASC-seeded PLGA scaffolds, compared to mice treated with scaffolds alone.\(^{41}\)

### 3.3 Induced pluripotent stem cells

Most recently, pluripotent cells originating from adult tissues, now coined induced pluripotent stem cells (iPS), were described by Takahashi *et al.* in 2006.\(^{42}\) Observed to have similar pluripotent capacity and cellular characteristics as hESC, iPS cells have been until recently derived from embryonic and adult postnatal fibroblasts reprogrammed to express Oct4, Sox2, c-Myc, and Klf4. Regarding their translational potential, advocates of iPS cells believe that these cells hold the potential to harness all of the positive attributes of hESC, while largely circumventing the aforementioned political and ethical concerns surrounding their study and clinical use. However, despite the substantial exuberance that has surrounded their development, iPS cells are not without their critics. The need for retroviral or lentiviral transfection in order to induce transcript expression, and the associated risk of viral integration into the recipient genome, pose significant concerns to application in humans. As such, efforts have ensued to derive iPS cells utilizing non-viral means, and moderate success has been appreciated. Okita *et al.* observed the successful production of
virus-free iPS cells from embryonic fibroblasts through repeated transfection of two independent plasmids containing complementary DNA sequences of Oct3/4/Sox2/Klf4 and c-Myc. Additional concerns have been raised regarding the extended period of ex vivo culture required for both adequate numbers of fibroblasts harvested from skin to be obtained for iPS derivation, as well as the time required in culture for the formation of iPS colonies arising from fibroblasts to be observed. The period of culture that has been observed to date for the iPS colonies to be generated post-transfection is currently on the order of weeks. Furthermore, the relatively low efficiency of transformation of fibroblasts to iPS cells, as well as the need to be cultured on a feeder layer of mouse cells, have presented additional concerns regarding the realistic feasibility of clinical translation. Thus, our group and others have begun to probe the potential of alternate adult cell populations to achieve this goal more expeditiously and efficiently. To this end, collaborative efforts between the Wu and Longaker Laboratories at Stanford University have put forth exciting progress on this front. Sun et al. have now reported that deriving iPS cells from human ASC (hASC-iPS) can be achieved in significantly less time (16 days vs. 28 days) and with substantially greater efficiency (0.2% vs. 0.01%) relative to their fibroblast counterparts. Furthermore, although with reduced efficiency, hASC-iPS can be derived and maintained in culture in the absence of a feeder layer of cells; additional properties not previously appreciated when deriving iPS cells from embryonic or adult fibroblasts. Such advances are potentially of paramount importance to the future of complex skeletal reconstructive scenarios. Through the application of truly pluripotent progenitor cells, the potential to address reconstructive needs involving not only multiple mesenchymal-derived tissue types, but tissues derived from all three germ layers, becomes increasingly feasible.

4. Pro-osteogenic Molecular Biology

Today, it is believed that successful transition of cell-based skeletal tissue engineering strategies to the bedside will necessitate the application of targeted molecular manipulations of osteoprogenitor cells in order to maximize their osteogenic potential and skeletal regenerative capacity.
Furthermore, an additional goal of delivering pro-osteogenic small molecules and cytokines via reconstructive constructs will be aimed at enhancing the endogenous regenerative capacity of surrounding native tissues, as well as incorporation of implanted reconstructive constructs with these tissues. Heretofore, presented is a current working knowledge regarding the molecular signaling pathways they have been identified as playing an important role in skeletal developmental biology, fracture healing, and the osteogenic differentiation of osteoprogenitor cells.

### 4.1 Bone morphogenetic protein

Of the important pro-osteogenic signaling pathways that have been identified, BMP has been most widely examined. To briefly provide some background, BMP is a member of the transforming growth factor-beta (TGF-β) superfamily. To date, more than 14 types of human BMP have now been described.\(^{45}\) Of these, BMP-2, -6, -7, and -9 have been observed to promote robust osteogenesis \textit{in vitro} and \textit{in vivo}.\(^{45}\) BMPs function in a dose dependent fashion, and demonstrate the greatest pro-osteogenic effects when present as the heterodimers BMP-2/6, -2/7, and -4/7.\(^{46,47}\) Regarding the osteogenic differentiation of multipotent mesenchymal precursor cells, BMP signaling has been observed to be mediated through Type I or II receptors, resulting in subsequent downstream activation of SMAD transcriptions factors through phosphorylation, and modulation of the transcription factor Cbfa1/Runx2.\(^{45}\)

Multiple investigations have highlighted the importance of BMP signaling to the osteogenic differentiation of both mouse BMSC and ASC.\(^{48,49}\) Furthermore, investigations by Panetta and Gupta \textit{et al.} in the Longaker laboratory have made strides in demonstrating the importance of BMP signaling to the osteogenic differentiation of human ASC. This work has been able to clearly define that human ASC respond to BMP in a dose dependent fashion. Furthermore, by means of qRT-PCR and ELISA, it was elucidated that human ASC display increased BMP gene expression and protein elaboration during the course of osteogenic differentiation. The specificity of this pro-osteogenic response to BMP was clarified utilizing BMP-2 specific neutralizing antibodies. Here, osteogenic differentiation was impaired in the presence of neutralizing...
antibody to BMP-2, and could be rescued through exposure to exogenous protein. These observations are critical, as they increase the viability of targeted molecular manipulations of BMP signaling, and potentially that of its antagonists, to enhance the osteogenic capacity of human ASC for use in cell-based skeletal tissue engineering strategies. In vivo, Cowan et al. observed augmented bony healing and bone turnover in critical sized 4 mm murine calvarial defects reconstructed with osteoblasts, BMSC, and ASC stimulated with rhBMP-2 ex vivo and seeded onto apatite coated poly-lactic-co-glycolic acid scaffolds. Encouraged by these in vitro and in vivo observations, considering their potential translational implications, ongoing studies are aiming to probe whether augmented BMP signaling has the capacity to enhance in vivo healing mediated by human ASC.

Speaking to the potential translational importance of BMP to bone tissue engineering efforts, clinical applications of BMP to augment endogenous skeletal healing have already achieved success in the clinical arena. FDA approval was first granted in 2002 for the delivery of rhBMP-2 on a Type I collagen sponge to treat of degenerative lumbar disk disease. Subsequently, preclinical trials by McKay and colleagues demonstrated that 94.5% of rhBMP-2 treated patients, compared to 88.7% of autograft treated patients, demonstrated disk fusion radiographically two years post-intervention. Today, the application of rhBMP-2 in the treatment of tibial fractures has also been approved for clinical use. Collectively, both these and the aforementioned observations strongly support the potential of augmented BMP signaling to enhance both endogenous and cell-mediated healing of bony defects.

4.2 Wnt

Recently, evidence that supports the previously unappreciated role of Wnt signaling to the osteogenic differentiation of mesenchymal progenitor cells has been put forth. Briefly, Wnt signaling is mediated by the binding of Wnt ligands, comprised of multiple secreted glycoproteins, to the transmembrane receptors Frizzled/LRP5/6 co-receptors. Upon binding, phosphorylation of Disheveled protein occurs. Next, through a complex interaction between Axin, Frat-1, and APC tumor suppressor, phosphorylation of β-catenin by GSK-3β is inhibited. This leads to subsequent
cytoplasmic accumulation of \( \beta \)-catenin, nuclear translocation, and transcription of downstream target genes. Regarding the role of Wnt signaling in the osteogenic differentiation of MSC, Gaur et al. demonstrated that Wnt signaling lead to the activation of Runx2 in mesenchymal osteoprogenitors.\(^{52}\) Additionally, Day and colleagues described that \( \beta \)-catenin mediated Wnt signaling enhanced osteogenic, and inhibited chondrogenic, differentiation of MSC.\(^{53}\)

**4.3 Fibroblast growth factor**

The family of FGF ligands consists of 23 related proteins, and signal transduction is mediated by four tyrosine kinase transmembrane receptors. Speaking to the important role FGF signaling plays in bone homeostasis, FGF-2 null and haploinsufficient mice have been observed to express reduced levels of FGFR-2 and Runx2, with resultant decreased bone density, relative to wild type mice.\(^{54}\) In an effort to clarify the specificity of these observations as they related to a decreased presence of FGF-2, subsequent studies observed that the osteogenic capacity of osteoblasts harvested from null and haploinsufficient mice was rescued in vitro by the addition of exogenous FGF-2 ligand. The important role of FGF signaling in the osteogenic differentiation of mesenchymal cells has also been described. Important to the identification of osteogenic signaling pathways in ASC that may potentially be amenable to targeted manipulations aimed at enhancing the osteogenic potential of these progenitor cells, Quarto et al. recently described the dynamic expression profile of FGF-2, -4, -8, and -18 in vitro during the course of mouse ASC osteogenic differentiation.\(^{55}\)

**4.4 Hedgehog**

The important role of the Hedgehog family of proteins in the process of skeletal development and osteogenesis has been observed. Although contributing in distinct fashions, two of the three constituent family member proteins (Sonic Hedgehog, Indian Hedgehog, and Desert Hedgehog) are now known to contribute to the regulation of bone formation. Sonic Hedgehog (Shh) plays an integral role in the process of skeletal
patterning. Alternatively, Indian Hedgehog (Ihh) expression is critical to endochondral ossification. During the activation of these proteins, Hedgehog proteins are cleaved from their secreted form to a 19 kDa N-terminal fragment. Active fragments then function in a paracrine fashion through binding to the cell surface receptors Patched (Pct) and Smoothened (Smo). Upon binding, Smo is released from the complex, allowing for the activation of downstream signaling factors Gli1, 2, and 3 and expression of Hedgehog target genes. Speaking to the importance of this cascade of events to the differentiation of osteoblasts, Hu et al. demonstrated that interruption of Ihh in primitive osteoblasts at a point in differentiation prior to the expression of Colα1, alkaline phosphatase, and Runx2, resulted in the arrest of the differentiative process. Having made this observation, ongoing studies are aiming to elucidate the role of hedgehog signaling in multipotent progenitor populations with potential for application in tissue engineering strategies.

4.5 Hypoxia-inducible factor 1-alpha

A growing body of evidence continues to illuminate the importance of angiogenesis in the process of skeletal regeneration and healing. Participating in this complex process, Hypoxia-inducible factor 1-alpha (HIF-1α) is now known to be critical to successful osseous healing. Wang and colleagues observed that HIF-1α over expression in osteoblasts of developing bone lead to increased levels of VEGF, resulting in highly vascularized bones with high bone mineral density. Additionally, and of significant importance to cell-based tissue engineering efforts, Cetrulo et al. demonstrated that systemically administered progenitor cells preferentially localized to ischemic regions of osseous healing, where HIF-1α upregulation was observed. This observation contributes additional support to further investigate the potential of this signaling mechanism to augment skeletal regeneration mediated by MSC.

In the future, simulation of angiogenesis will likely play a critical role in successful application of cell-based bone tissue engineering constructs in the operating suite. This is secondary to the fact that constructs studied to date have been limited in size do to their avascular nature, making cell survival dependant on diffusion of nutrients from surrounding tissues.
Through the incorporation of pro-angiogenic signals, it is the hope of surgeons that the viability of larger constructs will be appreciated, increasing the scope of potential clinical applications.

5. Advances in Skeletal Tissue Engineering Scaffolds

Although progenitor cells hold substantial potential to produce functional regenerated skeletal tissue on a scale not previously appreciated, they lack the capacity to put forth the necessary mechanical stability important to skeletal reconstruction during the process of ossification and the maturation of regenerated bone. Providing such mechanical stability is critical, while at the same time possessing degradation kinetics that allow for the timely replacement of the scaffold material by regenerated bone. Furthermore, as alluded to in our previous discussion, the utilization of pro-osteogenic and angiogenic small molecules and cytokines to augment the regenerative process will likely be a critical component of future reconstructive constructs. However, to appreciate optimal outcomes and avoid untoward effects of therapy, it will be imperative to deliver pro-osteogenic small molecules and cytokines in a very specific spatiotemporally controlled fashion to both progenitor cells and surrounding endogenous tissues. It is these factors that largely continue to guide the efforts of material scientists developing scaffold materials with an architecture and composition the successfully meets the needs of the reconstructive surgeons and patients requiring surgical intervention.

5.1 Scaffold composition

The composition of scaffolds is maybe the most important factor that affects their osteoinductive potential. In the broadest sense, scaffold materials can be divided into four categories: (1) natural scaffolds (i.e. collagen, hyaluronic acids, calcium alginate, chitosan, fibrin, thermoplastic starch biodegradable plastics, etc.); (2) mineral-based scaffolds (i.e. calcium phosphate ceramics, bioactive glass, etc.); (3) polymer scaffolds (i.e. polylactic acid, polyglycolic acid, polylactic co-glycolic acid, polydoxanone, polycaprolactone, polyfumarate, etc.); and (4) hydrogels. As each of these scaffold materials bring to the table specific strengths
and weaknesses, the advent of composite scaffolds has ensued. Examples of such composites include hydroxyapatites/polymers, calcium phosphates/polymers, and bioglasses/polymers. Here, efforts have been made to draw upon the positive attributes of each component material, while mitigating insufficiencies appreciated individually. Investigation probing the capacity of composite scaffolds to achieve this end have been encouraging. Lastly, material properties specific to hydrogel scaffolds, including their capacity to undergo temperature dependent phase change, in addition to the potential to have specific control over their chemical, material, and three-dimensional characteristics, collectively make hydrogels very attractive candidate scaffold materials for tissue engineers. With such malleability in composition as is seen in hydrogels, very specific control over the incorporation of small molecules and cytokines, as well as of their release through controlled degradation, becomes increasingly realistic.

5.2 Scaffold structure

In so much as scaffold composition dictates the osteoinductive properties of a scaffold, scaffold structure largely defines its osteoconductive characteristics. Innate to defining the osteoconductive properties of a scaffold are its capacity to allow for the integration of implanted osteoprogenitor cells with surrounding endogenous skeletal tissue to regenerate bone that mirrors endogenous tissues in form and function. Several scaffold structural characteristics have been identified that can significantly alter their capacity to achieve this end. Degree of porosity, pore and/or tube size, and resultant surface to volume ratio all represent structural properties that can affect the ability of a scaffold to direct neovascularization and nutrient delivery to regenerating bone. If inadequate, these factors can have a substantial negative impact on cell-mediated osseous healing.

5.3 Scaffold fabrication techniques

With a growing body of knowledge in hand informing tissue engineers of scaffold compositions and structures that may best guide osseous regeneration, the challenge becomes to produce scaffolds possessing these
properties in a reproducible and efficient manner. To this end, significant progress has been appreciated secondary to the development of novel fabrication technologies. Bioengineers and material scientists have set forth to develop techniques capable of generating scaffolds with a microarchitecture that closely resembles that of endogenous skeletal extracellular matrix. It is believed that doing so will help to guide progenitor cells to deposit matrix in an arrangement that mimics that of endogenous tissues. Here, current efforts are being focused on refining nanofiber technologies (photolithography, electrospinning, laser patterning, self-assembling peptides). Most recently, self-assembling peptide nanofiber scaffolds have garnered significant attention from scientists. Undoubtedly, clarifying all of the compositional and structural scaffold characteristics that will optimize skeletal regeneration, as well as the development of technologies that enable such scaffolds to be produced in a predictable fashion, will be critical for delivery of skeletal reconstructive modalities to the marketplace.

6. Summary and Future Directions

Undoubtedly, significant progress has been made in expanding our understanding of all of the critical components of cell-based skeletal tissue engineering constructs. Yet, for these technologies to someday become a reality and achieve translation to the bedside, as substantial amount of work to address unanswered questions remains. First, harnessing the capacity to define MSC subpopulations that may exist within the population of cells isolated by current techniques has remained elusive. It is certainly plausible, if not even probably, that the current population of cells we define as MSC is in reality a “minestrone soup” of progenitors with varying capacity for differentiation toward the various mesenchymal lineages. Identifying the unique cell surface antigen profiles of progenitor subpopulations may play a critical role in isolating osteoprogenitors with the greatest osseous regenerative capacity.

Second, the need for continued evolution of current scaffold technologies is unquestionable. We have now reached a point where future progress must focus on the development of increasingly biologically active scaffold materials. A wealth of knowledge is now in hand regarding
molecular signaling pathways critical to the osteogenic differentiation of multipotent osteoprogenitors. As such, novel scaffold materials must have the capacity to deliver small molecules and cytokines in a specific spatiotemporally controlled fashion, altering the agonist/antagonist relationships involved in the regulation of these pathways in a pro-osteogenic fashion. Additionally, the goal of such interventions should not only be to augment the osteogenic capacity of implanted progenitor cells, but simultaneously enhance the endogenous regenerative capacity of surrounding native bone. The envisioned end point of such manipulations would be the rapid and efficient production of a robust skeletal regenerate. With due diligence, success in addressing these needs, as well as needs that remain unrecognized, will assuredly come to fruition. In turn, such advances will make possible the realization of improved patient outcomes through the translation of skeletal tissue engineering to the bedside.

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1. Introduction

We have observed a rapid evolution in both surgical techniques and materials utilized in the promotion of regenerative therapy for oral reconstructive procedures. In particular, bone augmentation has been promoted through different methods which include the use of growth and differentiation factors, particulate and block grafting materials,\textsuperscript{1–4} distraction osteogenesis,\textsuperscript{5–7} as well as membrane-assisted guided bone regeneration (GBR).\textsuperscript{8} A central theme to all of these oral reconstructive technologies is the need for rapid and complete revascularization.

Early research that introduced us to the concept of using barrier membranes in what is referred to as GBR outlined the need for exclusion of undesirable soft tissue cellular contents and provision of a secluded space into which osteogenic cells from various sources can migrate for successful bone healing.\textsuperscript{9–11} The pattern of bone regeneration involves angiogenesis and ingress of osteogenic cells from the defect periphery towards the center to create a well-vascularized granulation tissue. This provides a
scaffold for woven bone proliferation and bone apposition within the defect. The size of the defect influences the bone healing capacity. In circumstances where the defect size is too large to generate a biomechanically stable central scaffold, bone formation will become limited to the marginal stable zone with a central zone of disorganized loose connective tissue. Critical to the outcome of GBR is maintenance of primary wound closure throughout the healing period.

Perforation of the cortical bone layer has been advocated in GBR as it has been postulated that this will increase the vascularity of the wound and release growth factors and cells with angiogenic and osteogenic potential. Aggressive recipient bed preparation with decortication, intramarrow penetration has also been supported due to increases in the rate of revascularization, the availability of osteoprogenitor cells and the increased rate of remodeling. In addition to the surrounding bone, the periosteum is also widely considered as an important source of cells with osteogenic capability. Despite the desirable graft containment and cellular exclusion characteristics of most barrier membranes, limiting the access of periosteal-derived osteogenic cells during the early phases of wound healing may not be of benefit to the healing of larger sized defects.

The autograft, allograft, alloplast and xenograft materials all have reported success either alone, or in combination, for particulate bone augmentation. The particulate autograft is currently recognized as the gold standard for most bone grafting, including the treatment of dental implant related defects. Several studies have demonstrated the effectiveness of particulate autograft due the availability of cells with osteogenic potential, as well as osteoinductive and osteoconductive properties. However, autografts have recognized limitations, such as donor site morbidity, increased cost, potential resorption, size mismatch, an inadequate volume of graft material, as well as the unpredictability in the quantities of osteogenic precursor cells.

Allografts have the advantage of being available in higher quantities and eliminate the morbidity associated with a second surgical site. Biochemical extraction techniques have shown that growth and differentiation factors are present in demineralized freeze-dried bone allograft (DFDBA) preparations; however, quantities have been shown to be variable from lot to lot indicating a potential variation in performance.
Thus, allografts primarily act as a scaffold for the in-growth of capillaries, peri-vascular tissues, and osteoprogenitor cells from the adjacent recipient bed.

The absence of differentiating precursor cells or osteoblasts in adequate quantities will ensue in limited bone formation. Osteoblasts contain the cellular machinery for production of bone matrix, but they are unable to undergo further division and have limited migratory capacity. This limits the expected benefits of cells contained in oral derived autografts, for example, which exhibit high variability in the numbers of cells with osteogenic potential.

Based on our current understanding of graft healing and the prerequisites for optimal bone regeneration, tissue-engineering research has been focused on providing the necessary cellular machinery, namely the mesenchymal stem cells (MSCs) and osteoprogenitor cells, directly in sites that require bone regeneration. It is this concept that has been utilized in the processing of the commercially available graft material that will be discussed here. Historically the majority of efforts for bone grafting with MSCs and osteoprogenitor cells have focused on the concept of harvesting these cells followed by in vitro culture expansion for later implantation.22,23 The following methodology section describes a novel approach that leaves the MSCs and osteoprogenitor cells found within allogeneic bone and substantially depletes unwanted cells.

2. Procurement Methodology for Stem Cell Containing Allograft

The allogeneic bone graft material described in this chapter (Osteocel®) is commercially prepared for NuVasive, Inc.™ from cadavers recovered by licensed tissue procurement agencies (AlloSource) and distributed into the dental market by ACE Surgical Supply. Cadaver tissues are rushed to the processing facility on wet ice and processing is begun within 24 hours of the donors’ death. In parallel rigorous safety testing, donor screening and evaluation for bacterial, fungal and spore contamination begins. Screening measures consist of physical examination and evaluation of both medical and social history, including a next of kin interview. Comprehensive serological and microbial testing are also performed
which includes nucleic acid testing (NAT) for Hepatitis-C and HIV. Donor assessment culminates with a complete medical record review by a licensed physician. Cortical bone is separated and processed into demineralized bone particles for adding back to the cellular graft component. A process of selective immunodepletion, that involves several extensive wash steps, is initiated to remove undesirable cells, such as red blood cells and lymphocytes that can provoke an immune response. These unwanted cells are substantially depleted leaving the remaining cell rich cancellous bone matrix. The cellular cancellous bone component then undergoes a broad-spectrum antimicrobial treatment (vancomycin, gentamicin sulfate and amphotericin) designed to eliminate potential contamination while preserving the viability of the cells. These remaining viable MSCs and osteoprogenitor cells remain attached to the cancellous bone matrix. Approximately 20% demineralized cortical bone particulate from the same donor is then combined to the cell containing cancellous bone matrix component. A standard cryopreservation solution containing 10% DMSO with human serum albumen (HSA) is added and the product is stored at −80 ± 5 degrees Celsius (°C), permitting a five-year shelf life.

During the product processing validation, FACS (fluorescence activated cell sorting) testing was performed to confirm the retention of MSCs that are positive for cluster of differentiation 105 (CD105) and CD166 while being negative for CD45.24 Figure 1 depicts a representative FACS Scatter Plot of the cellular allograft. While there is not a single identifiable surface marker for MSCs, this marker combination profile is indicative of MSCs and osteoprogenitor cells. Quality testing is performed on every lot of Osteocel® to validate a minimum cell count of 50,000 cells/cc, and a minimum cellular viability of 70% of the enzymatically released cells. Another iteration of the cellular allograft product (Osteocel® Plus), which was not utilized in the clinical and histological aspect of this chapter, has quality testing for a minimum cell count of 250,000 cells/cc. The cell count and viability are determined on released cells by a Trypan Blue dye exclusion test with a hemocytometer. Cellular osteogenic activity of each lot is also validated by performing in vitro cell differentiation and alkaline phosphatase assays (Fig. 2).

The cellular bone graft material is stored at −80°C, shipped to the clinic on dry ice where it is prepared as per the manufacturer’s recommendation.
Fig. 1. Representative FACS Scatter Plot of Osteocel®. (A) Forward Scatter and Side Scatter dot plot from the donor 2 population of Osteocel® Plus cells with [R1] 94.44%, [R2] 99.93% and [R1 + R2] 94.42%. (B) Dot plot showing positive expression of CD105 and CD166 markers, after gating for CD45- from the donor 2 cells. Quadrant gating UL 0.82%, UR 99.14%, LL 0.04% and LR 0.00%.

Fig. 2. Macroscopic aspect of the Osteocel® product. Following the appropriate thawing of the cellular allograft, there is a recommended four-hour window for its use. (Inset) Cellular image from the Osteocel® derived cells demonstrating positive osteogenic activity (staining positive for alkaline phosphatase activity).
Since the graft contains vital cells the maximum temperature of the water bath used during the thawing process should not exceed 37°C. After the cryopreserved cells are thawed, the liquid is decanted and the cells are rinsed with sterile saline. The cell containing graft is then ready for implantation, with a working window of four hours (Fig. 2). Depending on the defect treated, the particle size (1–3 mm) is often found to be too large for oral reconstructive procedures. In such instances rongeurs can be used to carefully reduce the particle size.

Scanning Electron Microscopy (SEM) images from this particulate graft have consistently revealed the cellular component of the allograft together with the extracellular matrix surrounding them. Figure 3 contains example SEMs at different magnifications. The SEM preparation starts with a 0.1M cacodylate buffer containing 5% sucrose rinse. The cells are fixed with 2.5% gluteraldehyde for one hour. Following three rinses with cacodylate buffer, the samples are soaked in 1% osmium tetraoxide solution in water for one hour at 4°C. The samples are subsequently dehydrated step-wise in increasing concentrations of ethanol starting at 50% and continuing to 100% for approximately two minutes at each step. A critical point dryer is used to dry the samples. The bone particles are attached to the SEM plates with adhesive and silver paint followed by a gold/platinum sputter coating prior to imaging. Images were captured utilizing a Quanta Model 600 (FEI, Hillsboro, OR) with a Tungsten filament at high vacuum mode and Soft Image Solutions (Olympus, Inc., Germany) software was employed for image collection.

3. Ridge Augmentation

With the greater acceptance and awareness of dental implant therapy as the strongest method of tooth replacement, amongst practitioners and patients alike, it is not uncommon to encounter reconstructive scenarios that require bone augmentation in the overall treatment plan. This is particularly seen in cases with long-standing edentulism, trauma and infection. Augmentation of the alveolar ridge for the ideal placement of an implant thus becomes necessary for an optimal esthetic outcome.25 The various techniques and materials employed in ridge augmentation procedures have been discussed elsewhere and is beyond the focus of this chapter.4 Of relevance, Osteocel®,
Fig. 3. (A) SEM image showing the cancellous bone coated with native cells. The cells are covered by extracellular matrix. (B) Higher magnification (2000x) of the box in (A). (C) Higher magnification (4000x).
as a cellular based grafting material, provides an attractive option for ridge augmentation, particularly in larger defects.

It is hypothesized that the cellular contents within Osteocel® would benefit from a rapid revascularization. This revascularization process takes place at a much faster rate from the periosteal source than bone. To exploit this notion, use of a classic barrier membrane should ideally be avoided, when possible, to facilitate this process during the initial stages of healing. Other graft materials containing molecular enhancement products have likewise been shown in certain studies to undergo a slower healing rate when used in conjunction with a barrier membrane.26,27 Figure 4 shows the successful use of Osteocel® alone for a small defect. The use of barrier membranes, however, may be deemed necessary when treating larger non space-maintaining defects for graft containment. In larger sized defects, as well as in cases where control of the location of the graft is critical, such as grafting against dental implants, a space-maintaining device in the form of a titanium mesh has been successfully employed.4,28,29

Titanium mesh offers resistance to bone graft collapse without the compromise in revascularization found with many products. However, when faced with a thinner tissue biotype caution is necessary when using any non-resorbable device as ensuing soft tissue dehiscences can lead to a compromised outcome.14 Recently Pieri and colleagues demonstrated titanium mesh use in combination with a mixture of intraoral autogenous bone and xenograft on 16 partially edentulous patients.30 They reported a mean horizontal augmentation gain of 4.2 mm. Only one of the cases showed early exposure of the mesh device. In cases where it was desired to regenerate more than 3 mm of horizontal bone we have predictably used the graft in conjunction with a titanium mesh for both space maintenance and graft containment preventing the cellular allograft from collapsing (Fig. 5).

4. Sinus Augmentation

The posterior maxilla represents an area that has historically posed a challenge for treatment with dental implants. These range from comprising sites with poor bone quality to unfavorable bucco-lingual resorption patterns and inadequacy in the vertical dimension of available bone following extraction of the teeth.31,32 In addition, bone regeneration within
the graft material is dependent upon it being populated by osteogenic cells that primarily originate from the osseous floors and walls, and to a smaller degree from the Schneiderian membrane. Thus, cellular infiltration, vascularization, de-novo bone formation and graft replacement often require long healing times to produce bone of adequate quantity and quality for implant placement in the posterior maxilla.

Fig. 4. Horizontal bone augmentation with Osteocel®. (A) Clinical view showing the dental implant dehiscence at the time of placement. Note intra-marrow penetrations have been made. (B) Clinical view with the cellular allograft in place. No membrane was utilized and the flap was sutured to tension-free primary closure with Vicryl™ sutures. (C) The four-month post-operative clinical view showing 2–3 mm of lateral bone augmentation and complete coverage of the implant threads. (D) A four-month post-operative CT scan showing the regeneration of a 2–3 mm thick buccal plate of bone over the implant.
Fig. 5. Horizontal bone augmentation with titanium mesh and Osteocel®. (A) Pre-operative clinical view. (B) Mucoperiosteal flap elevation revealing a deficient alveolar ridge in the horizontal dimension. Perforation of the cortical layer with intra-marrow penetration has been performed for revascularization of the Osteocel® graft. (C) A titanium mesh has been adapted and secured with screws for containment of the cellular allograft material. (D) The four-month post-operative view following titanium mesh removal revealing a 3–4 mm increase in horizontal ridge dimensions. (E) View of bone regeneration and Straumann implant placement after a partial reflection of the pseudoperiosteum that is often found when using titanium mesh. (F) Closure of soft tissues after implant placement, completely within the healed bone graft, showing a normal facial ridge profile.

Ongoing maxillary sinus pneumatization and normal post-extraction bone atrophy has been managed successfully by the sinus augmentation procedure either before or simultaneously with implant placement. The literature is inundated with reports describing this procedure using a variety of grafting materials. The use of a variety of materials has shown a varied bone quality and quantity with reported percentage bone areas that range from as low as 5% to over 40%.\textsuperscript{35,36} In addition, studies have demonstrated that it can take in excess of nine months to achieve optimal bone formation for implant stability.\textsuperscript{37,38}

Dental implant technology has endeavored for a faster osseointegration period to allow for more rapid restoration of the lost dentition. The concept of molecular enhancement of graft materials with either growth or differentiation factors for a more rapid regenerative outcome becomes desirable.\textsuperscript{39,40} Cellular enhanced bone graft materials potentially offer this timing advantage as well. Our experiences with Osteocel® as a sinus
augmentation bone graft material has consistently provided promising outcomes. This initial report based on histomorphometric analysis of grafted sinuses with Osteocel® showed an average vital bone content of 33% (range 22%–40%) and an average residual graft content of 6% (range 3%–7%) for cases that had an average healing period of 4.1 months (range from three to 4.75 months). These results were confirmed in a recent

Fig. 6. Sinus augmentation with Osteocel®. (A) Sinus access after a classic lateral window approach with simultaneous implant placement. (B) Sinus after grafting with the cellular allograft. No membrane was used to cover the bone graft and lateral wall access window. (C) A CT scan of the grafted sinus immediately after graft and implant placement. (D) A CT scan of the same grafted sinus after four months of healing. Note the radiographic evidence of increased bone density in the bone graft region surrounding the dental implant. The radiographic findings of significant bone formation are consistent with the histologic data showing average percent bone areas in excess of 30%.
Fig. 7. Histological evaluation of the healed bone after sinus augmentation with cellular allograft residual. (A) Representative mineralized histologic core of a cellular allograft residual grafted sinus. The portion of the core shown goes from the most superior aspect (left side) to the original sinus floor (right side). It was harvested four months following the cellular allograft sinus grafting procedure. The red stained tissue is either the residual mineralized cellular allograft material (lighter red, osteocyte nuclei not always visible) or newly formed bone (darker red, osteocyte nuclei visible). The green stained tissue is the residual demineralized allograft material (no cells visible, non-vital bone). New bone formation can be appreciated throughout the core. Bone has formed directly on the residual mineralized and demineralized allograft particulate as well as in areas without residual graft material (original magnification of 20×). (B) New bone of varying levels of
larger multicenter study.\textsuperscript{41} A faster graft healing time with respect to new bone formation in adequate quantities has encouraged an earlier initiation of implant placement and restoration. A sinus augmentation case with Osteocel\textsuperscript{®} is shown with the radiographic follow-up (Fig. 6) and histological evaluation after four months (Fig. 7).

5. Discussion

Stem cells can be derived from a variety of sources, including bone marrow, and are used in a variety of medical therapies. In the future, medical researchers anticipate being able to use technologies derived from stem cell research to treat a wider variety of systemic diseases, in addition to site specific repair as was described in this chapter. To optimize these exciting applications for stem cells a thorough characterization and understanding of stem cell biology will be required. Although stem cells can be isolated based on a distinctive set of cell surface markers, \textit{in vitro} culture conditions can alter the behavior of cells, making it unclear whether the cells will behave in a similar manner \textit{in vivo}. In fact, debate exists whether some proposed adult stem cell populations are truly stem cells. Because of their combined abilities of unlimited expansion and pluripotency, embryonic stem cells remain a theoretically viable source for regenerative medicine and tissue replacement after injury or disease. Differentiating embryonic stem cells into usable cells and ultimately organs is a challenge that tissue

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Fig. 7. (Continued) maturation can be appreciated in this higher magnification view (original magnification of 100×). (C) Osteoblasts can be seen lining the newly formed bone (original magnification of 200×). (D) Several areas of bone graft resorption can be appreciated in this view. This osteoclastic activity along with the low original packing density is likely responsible for the small percentage area that is the original cellular graft material. Multiple multinucleated cells can be seen (original magnification of 200×). (E) The difference in the level of bone maturity with areas of immature woven bone (right side) and areas of mature lamellar bone (left side) can be appreciated (original magnification of 100×). (F) Previous field (E) seen under polarized light. The demineralized bone areas (green) did not refract the polarized light to the extent the mineralized bone areas did. The difference in the level of bone maturity with areas of immature woven bone (right side) and areas of mature lamellar bone (left side) can also be appreciated with respect to the level of polarized light refraction (original magnification of 100×).
engineering researchers will face for years to come. Additionally, the use of embryonic stem cells is more controversial than adult stem cells.

Stem cells and progenitor cells act as a repair system for the body, not only replenishing specialized cells, but also maintaining the normal turnover of regenerative organs and tissues. In spite of this important function, pluripotent adult stem cells are rare and generally small in number within the body, with most being lineage-restricted (multipotent). They have also been shown to decrease in number with age. Bone marrow contains numerous cell types from both the hematopoietic stem cell lineage (for example platelets, osteoclasts) and the non-hematopoietic stem cell lineage (for example MSCs, osteoblasts). During the Osteocel® procurement procedures, immunogenic cells and tissues are substantially depleted. The potential for immune response is why typical fresh-frozen bone allografts are not as attractive of a grafting option, even though in a few reports success for oral reconstructive procedures has been demonstrated. One recent study on 21 patients reported high success with dental implant placement into fresh-frozen bone allograft regenerated bone. Since only a disinfection process is performed with typical fresh-frozen bone allografts and not a complete immunodepletion process, there are likely large numbers of undesirable cells remaining in the graft. This could potentially impact the graft performance and the host immune response.

For multiple reasons MSCs enjoy a hypoinmunogenic host response. MSCs lack MHC-II and co-stimulatory molecule expression. MSCs are also immunomodulatory in that they prevent T-cell responses indirectly through modulation of dendritic cells and directly by disrupting NK as well as CD8+ and CD4+ cell function. MSCs also induce a suppressive local microenvironment through cytokine production (prostaglandins and interleukin-10) and expression of indoleamine 2,3-dioxygenase which depletes the local milieu of tryptophan. One of the major proteins produced by MSCs is transforming growth factor-beta (TGF-beta) that regulates the host T-cells by promoting T-regulatory cells.

The cellular content of autogenous bone grafts varies based on the individual patient’s medical profile, the harvest technique (aspiration or open harvest), anatomic location of the harvest (intra-oral or extra-oral), type of bone harvested (cortical or cancellous), age and gender. The cellular
content has also been shown to have an effect on the bone graft performance. Therefore, the identification of MSCs and osteoprogenitor cells and determination of their concentrations in different anatomical tissues has been an area of recent investigation.\textsuperscript{46-48} Evaluation of bone marrow aspirates from the anterior iliac crest revealed a fairly small count of MSCs, although a higher percentage of cells that tested positive for CD105 was found in the iliac crest aspirates when compared to peripheral blood.\textsuperscript{48} McLain and colleagues compared the osteoprogenitor cell concentrations between iliac crest and vertebral body aspirates.\textsuperscript{46} Their findings show that vertebral aspirates (465 cells/cc marrow) have a slightly higher mean concentration than the iliac crest aspirates (356 cells/cc marrow). The process used to prepare the cellular allograft bone matrix described in this chapter involves the selective removal of immunogenic cells in hematopoietic lineage from cell-rich cancellous bone, while retaining the osteopotent cells in the mesenchymal lineage. The minimum number of MSCs and osteoprogenitor cells found in the commercially available Osteocel\textsuperscript{®} product is 50,000 cells/cc and for Osteocel\textsuperscript{®} Plus product is 250,000 cells/cc. Clearly these cell counts are a dramatic improvement over the cell counts for aspiration harvests and may result in an enhanced clinical result. Recently Cuomo and colleagues have conducted a preclinical trial evaluating MSCs from human bone marrow aspirates in combination with demineralized bone matrix in athymic rat femur critical size defects.\textsuperscript{49} Unprocessed MSC concentrations were found to vary from 64 to 2933 cells/ml with an average of $1010^{\pm}960$ cells/ml as determined from fibroblast colony forming unit (CFU-F) culture assays. MSC enriched bone marrow aspirate (centrifugation concentration) improved the yield to an average of 6150 cells/ml. Interestingly, the bone forming capabilities were found to be only comparable to the demineralized carrier alone. The authors mention the cell number may be insufficient to stimulate a robust bone formation. The clinical ramification of cell number has also been discussed by Hernigou and colleagues when treating tibia non-unions with marrow aspirates. They have identified 30,000 cells/ml as the minimum number of progenitor cells necessary to induce healing in this indication.\textsuperscript{50} Another possibility is that since the MSCs have not had a chance to adhere to the carrier some may wash out of the wound further reducing the MSCs
influence on the healing. All of these considerations could have clinical implications when performing oral reconstructive surgery with bone marrow aspirates as has been presented by some clinicians. 48

This exciting new cellular allograft technology will further assist us in the management of challenging oral regenerative procedures. Ongoing research is aimed at optimization of the clinical techniques and determining the long-term success of their application.

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References


1. Introduction

Spinal cord injury (SCI) is a devastating condition that affects 10,000 to 12,000 new individuals per year in the United States. In a recent survey by the Reeve Foundation, chronic cases were estimated to be 1,275,000. The current standard of care includes the optional use of high dose methylprednisolone,\textsuperscript{1} optional surgical stabilization and decompression,\textsuperscript{2} and rehabilitation.\textsuperscript{3} Recovery after SCI occurs, but is limited. Unlike the more progressive degenerative diseases, SCI affects mainly younger, healthy individuals, although this is changing, with increasingly more elderly people being injured, especially from falls.\textsuperscript{4} Since SCI involves both acute and progressive secondary injury to the CNS that results in neuronal and glial cell loss, cellular replacement therapies have long been sought for this condition.\textsuperscript{5} With the advent of modern stem cell biology, it is obvious to ask whether stem cell technology can be brought to bear on this problem, and many laboratories are pursuing this goal in experimental models of SCI. However, it is not so obvious what the targets of such
therapies might be. While axonal regeneration has been the holy grail of SCI research, strategies aimed at reducing secondary injury and inflammation and replacing myelinating oligodendrocytes are also clear targets for repair. Since there have been a number of recent attempts to use cell-based therapies in human SCI, it is important to have as clear a notion as possible as to the types of cells, and types of targets that might be useful and, “to do no harm.” Here, we will briefly review the biological features of SCI that might be amenable to stem cell therapy, and consider some of the approaches to therapy based on current knowledge of stem cell biology. Rather than a comprehensive review, we will attempt to identify some of the highlights of recent work in this field, and the problems and promise that they present.

The use of the terms stem and progenitor cells in this article need some definition, as they are often differently applied. Stem cell is meant to include cells from embryonic, fetal, or adult organisms, including humans, that are pluripotent and self-renewing. Progenitor cells represent cells that have more limited potential, usually limited to cells of a single germ layer. In the CNS, these are usually referred to as neural progenitors. Neural progenitors (or multipotential precursors) can divide and differentiate into lineage specific progenitors, including neuronal and glial progenitor cells. Of course, CNS repair also involves cells originating from all germinal layers (e.g. endothelial cells, immune cells like microglia, etc.). The use of human embryonic stem cells per se has actually been somewhat limited in this field, but there is much to be learned from the use of other cell-based therapeutic strategies that have used fetal or adult stem and progenitor cells from both humans and animals. There may be diverse sources for stem cells that eventually will be applied to clinical use. Very recent advances in re-programming human somatic cells to produce the so-called inducible pluripotent stem cells (hIPSCs) point to the rapid changes in stem cell technology that are likely to affect clinical uses. Thus, we will consider data from multiple experimental paradigms using a variety of stem and progenitor cell types, since it is possible that the features of each of these phenotypes could be generated from multiple origins. The ideal cell, perhaps, would be an autologous, pluripotent stem cell
population that could be conditioned to form any of the many parts of the mature CNS without forming tumors.

2. Secondary Injury and Endogenous Repair After SCI

Experimental studies of spinal cord injury (SCI) have made significant advances in recent years with the development of multiple approaches to modeling the human condition, and real advances in the understanding of secondary injury and repair. It is now well established that the initial injury is followed by a cascade of events that exacerbate the primary lesion via excitotoxicity, components of acute inflammation, and longer term processes that can result in apoptosis of myelinating oligodendrocytes that may contribute to demyelination and loss of function. It is also clear that the sequelae of SCI and other CNS injuries include regenerative and reparative processes that tend to restore or protect the remaining CNS, including the proliferation of endogenous progenitor cells, and the walling off of the injured zone by astroglial hypertrophy. It is somewhat difficult to categorize many of these post-injury events as either positive or negative with respect to the final functional outcome. Thus, while the astrocytic response to injury may be protective, as is scar formation in peripheral tissues, the glial scar presents a serious impediment to axonal regeneration. Similarly, acute inflammation is thought to be destructive to oligodendrocytes, and yet aspects of the cytokine response appear to promote oligodendrocyte precursor proliferation and differentiation. There appear to be many similarities between the cellular events leading to demyelination and conduction failure in multiple sclerosis (MS) and in SCI, and in both cases, a reparative response of the resident precursor population is mounted, but is insufficient to provide complete remyelination and recovery. Microglial activation is a prominent feature of the early injury response in rats and humans and, like the more general concept of inflammation, microglial activation has been associated with both cellular degeneration and repair. These mixed degenerative and regenerative events provide the background for targeting repair using exogenous stem or progenitor cell transplantation, that is, therapeutic targets for cellular transplantation can be defined by the biology of injury and repair (Fig. 1).
3. Therapeutic Targets for Transplanted Stem and Progenitor Cells

Targets include protection of cells that survive the primary insult (both neurons and glia), replacement of lost cells (neurons for re-establishing circuitry and glia for remyelinating spared axons). Exogenous glial cells can also provide a substrate for axonal growth and regeneration as well as providing growth and trophic factors that reduce long term secondary cell loss and enhance axonal regeneration and sprouting, and have been shown in some cases to reduce the endogenous glial scar.

The usefulness of animal models of spinal cord injury for preclinical testing of repair strategies depends upon their reliability and validity.\textsuperscript{19,20} In recent years, most studies of experimental SCI have used rodents, mostly rats, but increasingly mice to take advantage of current technology for genetic manipulation. More recently, a return to larger animal models in SCI has been discussed and initiated.\textsuperscript{21} Rodent models are convenient and relatively inexpensive, and, many would argue, that the basic biology of the lesion and the repair processes are similar in humans. However, there are many different ways to injure the spinal cord, and these may not always be similar to the injuries incurred in clinical settings. Thus, many laboratories use partial transection or complete transection injuries, even though most human injuries are more like the contusion and compression models that have been characterized extensively in the past two decades.\textsuperscript{22–24} The manner of injury will affect not only the normal patterns of recovery but also the strategies and utility of transplanting stem and progenitor cells (see Fig. 2). Complete transections of the cord have been used to test strategies aimed at true axonal regeneration, i.e. the regrowth of severed axons across a gap in the CNS (Fig. 2A). In this case, replacement cells must survive in the hostile lesion environment, integrate with the host tissue at the cut ends of the cord, and provide a tissue bridge and substrate sufficient for axons to grow across and reconnect with the caudal spinal circuitry. In addition, the glial scar, consisting of proteoglycans and hypertrophied astrocytes, along with multiple chemical signals repulsive to axonal elongation,\textsuperscript{15} extends across the entire access route to the gap. Despite this, axonal growth bridging a complete transection has been accomplished in a number of laboratories using either peripheral nerve grafts\textsuperscript{25} or using immature and proliferating Schwann cells in matrigel bridges,\textsuperscript{26} as well as grafts of fetal tissue containing stem and progenitor cells.\textsuperscript{5} And, there are reports of success with transplanted progenitor cells including olfactory ensheathing glial cells and bone marrow stromal cells.\textsuperscript{27} However, these dramatic anatomical reconnections have resulted in only modest if any recovery of function. In addition, there are practical issues with these experiments: the animals (usually rats) are severely disabled, lack bladder function for weeks, and require intensive nursing care.
Thus, these studies have been confined to a few laboratories. The fact that only a minority of human SCIs are in fact complete anatomical transections has also contributed to the rationale for not pursuing these difficult studies.

More laboratory studies have used partial transections, and as can be seen in Fig. 2, the lesion presents a less stringent situation for repair;
damaged axons can grow around the lesion, the glial scar is limited to one side leaving “normal” tissue as a substrate for growth, spared axons can sprout below the lesion, and the transplanted cells can provide trophic or growth factors to the adjacent tissue. Work from the laboratories of Mark Tuszynski at UC San Diego and the Drexel research group in Philadelphia have attempted repair of partial cervical cord transections using, fibroblasts engineered to produce neurotrophic factors, or bone marrow stromal cells and report both sparing of intact axons and the stimulation of growth around, and sometimes into, the transplanted lesion site. These studies provide for more “high throughput” results than complete cord transections or large contusion lesions, but there is always a question of whether functional effects are due to true regeneration, sprouting, or neuroprotection, and whether the results will translate to the contusion lesions most often seen in human SCI.

The contusion lesion (Fig. 2C) presents additional issues; it is more extensive than the cut lesions, affecting a larger area and potentially requiring significantly larger numbers of cells for transplants, but it nearly always has a rim of spared tissue (Fig. 3) that contains surviving axons which subserve spared function and which might be susceptible to damage from the transplantation process. This rim also could provide a physical substrate for regrowth and regeneration of cut axons. The spared rim can contain demyelinated axons, which may be dysfunctional due to conduction loss. These may be a target for therapies that is not available in the partial transection models. The glial scar in this case is also larger than in the transection models. The center of the lesion is frequently occupied by cystic cavities which contain rafts of macrophages especially early after the injury, but which also provide ideal receptacles for transplanted tissue. Recently, much more attention has been given to attempts to use cell-based therapies in this kind of model, with a variety of cell types and successes (see below).

In all of the models used, important questions involving the survival of the transplanted cells, the identification of host versus donor cells, the lesion microenvironment, and the role of inflammation and immune rejection need to be asked and answered. the answers may vary substantially depending upon the type of lesion used.
Fig. 3. Remyelination as a target for stem cell therapies. (A) Cross-section of the spinal cord at the lesion center three weeks after a 25 mm MASCIS injury in the rat. A spared rim of fibers can be observed to have survived the injury (arrow). This rim is separated from a forming cystic cavity by astrocytic processes that wall off the remaining tissue (double arrows). The central region of the injury site is occupied primarily by large numbers of phagocytic macrophages which have taken up the degenerating debris, and ingrowing cords of Schwann and mesenchymal cells as well as regenerating axonal sprouts from the adjacent dorsal roots (DR; see Ref. 32). (B–D) The spared rim of tissue (C, ventral funiculus) is also present after a contusion injury in the primate (500 gm-cm injury; ten-week survival). The central cavitated region contains macrophages (macs) and is walled off by astrocytic processes (double arrows in B). The remaining peripheral rim of white matter contains many smaller myelinated axons which have survived the injury interspersed with degenerating ones that have not yet been phagocytosed, and microcysts left by axonal and glial cell death. In the more central regions (D) closer to the cavity, there are few surviving myelinated axons and many demyelinated ones (large arrows) interspersed among astrocytic processes, macrophages (mac), and microcysts (MC). (Toluidine blue stained 1 μm thick sections; dorsal is up in all sections; A — 4X, B and C — 20X, and D — 63X.)
5. Types of Stem and Progenitor Cells Used for Transplantation in SCI

As noted, there is a long history of transplantation of tissue into the spinal cord using peripheral nerve tissue, fetal grafts, and other approaches, and Schwann cells have been used for many years to “bridge the gap” in studies of complete transections of the cord. Recent work suggests that these cells may be useful in combination with other treatments in complete cord transections (here using olfactory ensheathing glial progenitors), and in contusion injuries as well. Pearse et al. reported that Schwann cells in combination with cAMP treatments meant to stimulate axonal growth could impact both axonal growth through a contusion lesion, and have some effect on neurological recovery using several measures. While Schwann cells are not stem or progenitor cells, they can undergo proliferation in response to injury, and so constitute a cell that can be expanded and used for transplantation. Schwann cells can be taken from peripheral nerve biopsies and expanded, providing for a source of autologous cells for transplantation into chronic SCIs. Schwann cells also produce neurotrophic factors, and share many of the features of progenitor and stem cell populations considered elsewhere in this article. Schwann cells can also be derived from stem or progenitor cells. For example, mouse skin has been used as a source for neural crest-derived neurospheres and stem cells that can be treated in culture to produce stem-like cells, and these have been transplanted into spinal cord injuries. These cells, termed “SKPs”, differentiated into a Schwann cell-like phenotype (compared to neurospheres or “naïve” SKP cells) and transplanted after a moderate thoracic contusion injury in rats have been shown to (1) reduce lesion size (neuroprotection), (2) reduce the deposition of chondroitin-sulfate proteoglycans (CSPGs) around the injury cavity, (3) support the regrowth of axons from the spinal cord and brain, (4) increase the migration and proliferation of endogenous peripheral Schwann cells into the cord, and (5) have some, although modest, effect on neurological outcome. Such studies emphasize the complexity of interpreting cell-based therapies, but also suggest that the right cell type can act on several of the proposed targets.
Both mouse and human embryonic stem cells have been transplanted directly, or used as progenitor cells for deriving more restricted populations of cells for transplantation into mouse and rat spinal cord injuries. Cells derived from embryonic or fetal spinal cord and brain have been used as well, especially a population of cells isolated from the embryonic day 13–14 rat spinal cord that includes glial restricted precursor cells and neuronal restricted precursor cells (GRPs and NRPs). Human and rodent bone marrow-derived stromal cells (BMSCs) have been used extensively in spinal cord injury models and have also been used in initial human clinical trials. BMSCs can be delivered directly into the cord parenchyma, but have also been delivered by intravenous and intrathecal injections. There are a number of reports of neuroprotection and even transdifferentiation into neurons, but the generation of neural cells from BMSCs has been questioned. Timing of delivery may be particularly important when using BMSCs, with reports that acute delivery provides more neuroprotection than when cells are transplanted at one week or later. This is in contrast to reports of neural progenitors, which seem to flourish best when transplanted a week after injury. The use of BMSCs for transplantation in the cord is especially attractive because of the potential for using autologous cells, although most of the experimental work has used human or rodent allogeneic sources. This has been accomplished in a limited clinical trial with additional treatment with granulocyte colony stimulating factor (gcsf), and although the results of this trial are preliminary, this approach is likely to receive additional attention in the future. In addition, human BMSCs can be engineered to express growth factors or transcription factors like Olig2 that may enhance their usefulness in promoting recovery.

6. Evidence for Effects on Regeneration and Sprouting

Examples of reports of axonal regeneration across a complete transection injury after transplantation, with associated functional changes, include a study of transplanted olfactory ensheathing glial cell progenitors and a recent study in which olfactory ensheathing glia were paired with cAMP treatments and Schwann cell transplantions. In partial transection models, Davies et al., transplanted astrocytes derived from embryonic
glial-restricted precursors (GRP-derived astrocytes, GDAs) acutely into the hemisected rat cervical spinal cord and compared the results to “naïve” GRPs. GRPs survived and filled the lesion, but failed to provide support for growing transplanted dorsal root ganglion cell axons. GDAs, on the other hand, supported extensive growth of DRG axons across the lesion through an aligned network of astrocytic processes. Some of the recent studies using BMSCs have reported enhanced sprouting of axons, including the cortico-spinal tract.49

7. Evidence for Effects on Neuroprotection

Transplantation of stem or progenitor cells may affect lesion size and cell survival by reducing excitotoxicity and inflammation,13 or by providing trophic factors that reduce longer term apoptotic cell death.52,53 Transplantation of glial restricted progenitor cells in our lab reduced the apparent inflammatory response, and in addition, reduced the reactive glial scar surrounding a contusion lesion.54 Others have shown similar effects.55 Transplanted Schwann cells along with cAMP treatments reduced the inflammatory response to contusion lesion and appeared to result in enhanced neurological outcome.36

8. Evidence for Replacement of Neurons

McDonald et al.38 reported that transplantation of mouse embryonic stem cells nine days after contusion injuries resulted in the formation of human cell-derived astrocytes, oligodendrocytes, and neurons, with an apparent effect on locomotor outcome. Lepore and Fischer56 transplanted rat E14 fetal spinal cord, and mixed neuronal and glial progenitor cells isolated from fetal cord into partial hemisection injuries. These cells were taken from the hPLAP transgenic rat (as in Hill et al.54) so they could monitor cell survival and integration into the adult host. Many of the cells died within four days of transplantation, but those that survived proliferated to fill the hemisection cavity by three weeks, and included many donor-derived neurons. This group went on to transplant GRP/NRP combinations into the contused rat spinal cord nine days after injury,57 and reported not only reduction of lesion volume (i.e. neuroprotection), but
also the presence of mature glial and neuronal cells, and improved recovery of motor and bladder functions. Cummings et al.\textsuperscript{58} isolated human adult neuronal stem cells from neurospheres and transplanted them into contusion lesions in immune-compromised (scid) mice, and found mature neurons and glial cells, along with improved behavioral recovery. Yan et al.\textsuperscript{59} recently reported that human fetal spinal cord-derived stem cells produced both neurons and oligodendrocytes when transplanted into the uninjured or lesioned lumbar cord of nude rats. Many of these studies have used other cell types (e.g. fibroblasts) as negative controls, and the evidence for neural replacement using stem cells is mounting. Human spinal stem cells, produced from eight-week human fetal spinal cord tissue, were also used in a model of spinal cord ischemic injury,\textsuperscript{60} where they were found to replace lost GABAergic interneurons selectively lost to the ischemic insult. Thus, there is now ample evidence that neuronal replacement may be a useful strategy in treating spinal cord injury. Still needed are more clear demonstrations that these replaced cells can integrate into the host circuitry and affect useful functional recovery.

9. Evidence for Oligodendrocyte Replacement and Remyelination

Mouse embryonic stem cells, “neuralized” by treatment with retinoic acid (RA) were reported to produce neurons, astrocytes, and oligodendrocytes after implantation into the contused rat spinal cord nine days after injury.\textsuperscript{38} Further, these animals exhibited better neurological recovery on the “BBB” locomotor scale, showing plantar stepping with weight support as opposed to controls that scored about two to three points lower (no weight support or stepping). In a later study, this same group used similar cells, but with factors added to the culture medium that induce oligodendrocyte lineage differentiation (including tri-iodothyronine, T3) in transplants into the demyelinated dorsal columns (rats) or into the cord of \textit{shiverer} mice lacking normal myelin.\textsuperscript{61} These cells appeared to produce remyelination. Several reports cited above of mixed or undifferentiated stem and progenitor cells that reported neuronal generation and integration also reported oligodendrocytes from donor cells.\textsuperscript{57,58}
Kierstead et al. report on extensive experiments in which oligodendrocyte progenitor-like cells (OPCs) derived from human embryonic stem cells were transplanted into rat spinal cords after contusion injuries, produced myelinating cells, and appeared to enhance neurological function (i.e. a higher BBB locomotor score — two points), when transplanted at seven days, but not ten weeks, after contusion injury. These cells were derived from the H7 hESC lines at passage 32 via an extended (42-day) protocol in which neurospheres were isolated and exposed to thyroid hormone, FGF and other growth hormones. Purity, in terms of the presence of oligodendrocyte progenitor markers was reported to be above 80%. Cao et al. used GRPs that were genetically modified to express D15A, a mixed action neurotrophic molecule, to repair contusion injuries in rat cord, and found extensive remyelination by donor cells, accompanied by return of descending motor evoked potentials and improved behavioral recovery.

In our own laboratory, Hill et al. showed that GRPs from hPLAP transgenic rats could be acutely transplanted into large contusion injuries of the rat cord, and appeared to form myelinating cells as well as provided anti-inflammatory and neuroprotective effects. However, neither that study, nor a follow-up study transplanting GRPs at nine days after injury, produced substantial improvement in behavioral or physiological outcome measures. Thus, there is general agreement that GRPs and many other progenitor and stem cell types can survive and remyelinate the spinal cord. Obtaining consistent functional recovery in animal models may require optimization strategies, including the use of combination therapies. Nevertheless, remyelination has emerged as a major and near term target for cellular replacement therapy in spinal cord injury, and is being actively pursued as a clinical strategy.

10. Keys to Future Progress

Stem and progenitor cells would seem to be a logical therapy for replacement of lost glial cells and neurons, and many laboratories have used a variety of cell types from CNS and elsewhere in attempts to provide repair. Indeed, tissue transplantation has a long history in spinal cord injury, starting with Ramon y Cajal, and including the use of peripheral
nerve grafts, Schwann cells, and fetal tissue.\textsuperscript{26,34,64} It is only recently that it has been realized that in many of these studies, the transplanted cells or fetal tissue underwent massive cell death early after transplantation, and that the repair and replacement process was due to either replacement of exogenous cells by endogenous Schwann cells\textsuperscript{65} or appeared to involve die-back of the graft and repopulation of the host by proliferation of a small number of surviving donor progenitor cells.\textsuperscript{34} This was simply because most studies did not examine the acute and sub-acute fate of the transplanted cells, but rather, and understandably, heralded the presence of functional grafts in more chronic stages. In addition, the use of genetically-labeled donor cells has provided an easier method for tracking cellular transplants. The Hill \textit{et al.}\textsuperscript{65} demonstration of variable Schwann cell survival with different transplant times and immunological suppression, for example, used the transgenic hPLAP rat as a donor. There are many examples of failure to thrive after transplantation of precursor or progenitor cells, and it has been suggested that in many cases, the most multipotential cells are most vulnerable to early death, while more lineage-committed cells are more likely to survive.\textsuperscript{56} Thus the variable success of different cellular transplantation strategies may be due to biological features of both the host environment and the properties of the donor cells. More information on the survival, proliferation, and differentiation of transplanted progenitor cells after grafting is needed. The need for such information is driven not only by cellular transplantation studies in which there is a lack of enhancement of recovery,\textsuperscript{54,66} but even more so by remarkable hints at the prospects for dramatic success. Findings include evidence of enhanced function after transplantation of olfactory ensheathing cells, increased bladder and motor function after transplantation of mixtures of neuronal and glial restricted precursor cells,\textsuperscript{30} enhanced recovery with GRPs engineered to express neurotrophins,\textsuperscript{62} remyelination and recovery using oligodendrocyte progenitor cells derived from human embryonic stem cells (hESCs),\textsuperscript{39} remyelination and some recovery with neural precursor cells derived from adult subventricular zone,\textsuperscript{67} glial-restricted precursor-derived astrocytes implanted into partial cord transections,\textsuperscript{51} success with Schwann cell transplants if paired with cAMP elevations\textsuperscript{36} and many others. Many of these report that out of several treatment strategies, only one was successful; i.e. these are not just
positive reports, but also contain data suggesting that many strategies are ineffective. For example, the exciting report from the Fehlings lab\textsuperscript{67} reports that pilot studies showed that adult-derived neural precursors were not successful unless transplantation was accompanied by the application of growth factors, immune suppressants, and anti-inflammatory (minocycline) treatment. Further, a careful counting of cells in some of the subjects suggested that less than 40% of the number of transplanted cells remained after eight weeks; proliferation rates were examined late after transplantation and found to be low, but were not examined early. Thus, the actual percentage of cells surviving is not clear. In addition, while 50% of the surviving cells were positive for oligodendrocyte progenitor cells or mature oligodendrocyte markers, and myelination was seen, the remaining cells were astrocytes, or more often, unidentifiable by the markers used. And, enhancement of function was significant, but modest (i.e. about two points on average on the BBB scale). Thus, there is room for improvement even in this remarkable result. And, in contrast, the reports of a similar degree of success with OPCs derived from hESCs\textsuperscript{39} required little if any “help” from combination strategies. In summary, there is much to be learned about progenitor cell transplantation, and systematic studies of cellular interactions with the injury environment should help to improve the ability to predict which strategies will be most successful.

Further, there needs to be continued attention to improving preclinical studies and outcome measures, and an attention to possible adverse effects of cellular transplantation. As noted, the thin rim of demyelinated axons in contusion lesions may be a target for remyelination and a substrate for regrowth, but it also may be damaged during transplantation procedures. Another consideration is the cells providing growth factors that enhance repair mechanisms may also encourage the growth of sensory fibers and circuits that can contribute to spasticity and pain,\textsuperscript{68} or autonomic dysfunction.\textsuperscript{69}

11. Are Stem and Progenitor Cell Therapies Ready for Clinical Trials?

Given the questions above, it is pertinent to ask whether the field is ready to move into clinical practice. In truth, we are already there, with several
cell-based therapies being employed in SCI across the world. However, the issue involves identifying the pros and cons of moving cell-based therapies into controlled clinical trials in the US and Europe. The ProNeuron trial using activated macrophages, and the University of Florida experience with human fetal cell transplants at least show that these procedures can be accomplished without obvious harm. The technical problems of using stem and progenitor cells have been at least partially solved, but questions surrounding immunogenicity, tumorogenesis, and adverse effects such as chronic pain remain. Some would say that the potential benefits already outweigh the risks, and there are a number of planned trials including one focused on remyelination by oligodendrocyte progenitors derived from human embryonic stem cells (Geron, Inc.), which we have emphasized as a prime target in this article. Risk benefit analysis should also include consideration of the cell source and purity, and the route of delivery (e.g. injections versus open surgical approaches). The timing of therapy with respect to injury may also be important. There is much less positive data in chronic spinal injury models than in acute or subacute (within one to two weeks). Each case therefore should be determined on its own merits with the excitement of new therapeutic opportunities balanced by the axiom of “do no harm.”

Acknowledgments

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Potential of Tissue Engineering and Neural Stem Cells in the Understanding and Treatment of Neurodegenerative Diseases

Caroline Auclair-Daigle and François Berthod

1. Introduction

Neurodegenerative diseases consist of an heterogeneous assembly of pathological conditions in which specific regions of the central nervous system (CNS) relatively slowly and progressively deteriorate, resulting in movement and/or cognitive impairments. The specific etiology and causes of those disorders mainly affect elderly people. On the economic level, these types of neurodegenerative disorders are generating economical cost that sum up, each year, to hundreds of billions of dollars in developed countries. Given that the cause of most of these diseases remains unknown, the in vitro reconstruction of tissue-engineered models that mimic the diseases could be very helpful in understanding the subtle biological alterations that occur in comparison to the controls. In addition, since neurons lack the ability to regenerate, stem cells therapy may offer an opportunity to replace cells lost through damage or degeneration.
2. Neurodegenerative Diseases and Their Current Treatments

2.1 Parkinson’s disease (PD)

The second most common neurodegenerative disease, inflicting debilitating troubles to close to 1% of the population aged 60 and over. The suffering individuals are mainly affected by motor symptoms such as rigidity (muscle stiffness), bradykinesia (severe uncontrollable movement disorder), and shivers while resting. PD is a progressive disease characterized by a degeneration of dopaminergic neurons in the substantia nigra, and a subsequent deficit of dopamine release in the striatum. The affected individuals are becoming severely disturbed and depressed, in addition to the development of dementia over the span of the disorder (greater than 70% in advanced PD patients). There is currently no cure and no effective long-term treatment for PD, as the precise etiology of the neuronal loss is still unknown and the pathogenesis not fully understood.

The main treatment has been consisting, ever since the late 1960s, in the pharmacological lessening of the striatal dopamine deficit by administration of the dopamine precursor L-Dopa, which crosses the blood-brain barrier and enters neurons that convert it into dopamine. Alternative drugs were developed over the years, but failed to match the L-Dopa efficacy, which still remains the gold standard for the treatment of PD. However, its chronic administration is associated with motor complications reflecting fluctuations of the drug concentration in the plasma, and its efficacy lessens with time, with re-emerging of parkinsonian symptoms. Neurosurgical procedures have also been used as alternative approaches, consisting of ablative procedures of specific regions of the brain, deep brain stimulation using electrical currents, GDNF delivery in the striatum (a neuroprotective factor) through osmotic pump, slow-release beads or gene therapy, and cell replacement of the diseased neurons using fetal mesencephalic neurons.

2.2 Alzheimer’s disease (AD)

A progressive neurodegenerative disorder of the cortical regions of the brain, first affecting memory functions and then gradually affecting all
cognitive functions with behavioral impairments, leading to the irre-
versible loss of neurons and dementia. AD is the most common cause of
dementia worldwide, accounting for 50%–60% of all cases, some of its
risk factors including older age, family history, lower education level, and
female gender. In AD, some cholinergic neurons lose their ability to func-
tion, reducing acetylcholine level. Current treatments include the use of
drugs such as acetylcholinesterase inhibitors, in conjunction or not with
NMDA-antagonists, but they have not been shown to delay institutional-
ization or functional decline. Life expectancy following diagnostic
usually range between three to 15 years, but may be more limited.

2.3 Huntington’s disease (HD)

An autosomal dominant hereditary neurodegeneration initially described
by psychiatrist G. Huntington in 1872. It consists of a disorder primarily
affecting selective neuronal subtypes, and particularly GABAergic
medium spiny neurons, the main neuronal subtype in the striatum. The
mutant gene encodes the huntingtin protein, and the disease is believed to
be due to a gain of toxic function of the mutant protein. The prevalence
of this condition varies in the range of two to ten cases every 100 000, and
the onset of the disease usually occurs in people aged between 30 to 50,
but has also been observed in the elderly. At this point, there is a current
lack of effective treatment for HD. Since the abnormal huntingtin protein
and its cellular functions have been identified in 1993, drugs have been
elaborated to reduce the ailment’s magnitude. Strains of mice have been
created with the identical gene responsible for HD in humans, leading to
the development of promising drugs, such as drugs that block the gluta-
mine chains from clustering. The drug tetrabenazine proved to lower
dopamine release, limiting writhing movements.

2.4 Amyotrophic lateral sclerosis (ALS)

A progressive neurodegenerative disorder characterized by selective loss
of lower spinal and brainstem motor neurons and upper motor neurons. Of
the major physical consequences observed are paresis of skeletal and
bulbar muscles, amyotrophies, fasciculations, spasticity and ultimately,
paralysis. There appears to have no identifiable underlying cause other than genetic basis in familial cases, and there is currently no effective treatment available. The disease normally progresses rapidly, and survival rate rarely exceed three to five years after the onset. Death occurs as a result of diaphragm weakness, or from pulmonary infections. The only drug treatment currently approved for ALS is riluzole, a glutamate-release inhibitor. The drug possibly will expand life from four to 18 months and postpone the need for tracheostomy.11,12

2.5 Multiple sclerosis (MS)

An idiopathic primary demyelinating disease of the CNS, whose main condition generates destruction of normally developed myelin sheaths. It is a chronic progressive disorder characterized by disseminated neurological symptoms and, usually, several relapses during the course of its debut stage. MS was initially described by J. Charcot in 1866, defining the disease as a grouping of intention tremor, spastic paraplegia, speech impairment, visual loss and nystagmus. It currently stands as the most studied demyelinating disease. The most common theory for its presupposed etiology is multifactorial, stipulating that MS develops due to the combined presence of exogenous factors in genetically predisposed people, autoimmune response, and demyelinating lacerations in the white matter of CNS.9 This episodic neurological disease normally strikes people aged 20 to 40, although 10% are recollected in people aged over 50. Current treatment for relapsing-remitting MS are not curative, but include interferon-β-1a or interferon β-1b, and glatiramer acetate (namely copolymer 1), a combination of random polymers that mimic the amino acid composition of myelin basic protein, which could both be postponing the onset of major disability. Other pharmaceutical agents such as α-4 integrin antagonists, intravenous immunoglobulin infusions and corticosteroids may limit the relapse rate.13

3. Tissue Engineering as a Tool to Better Understand Neurodegenerative Diseases

As previously discussed, the causes of most neurodegenerative diseases do remain unknown. The usual method to analyze such disorders is to
perform histological and immunohistochemical analysis on post-mortem brain biopsies obtained from the patients, compared to normal tissues. These observations revealed the formation of $\beta$-amyloid plaques in AD, Lewy bodies in PD or superoxide dismutase-1 (SOD-1) aggregates in ALS, but they did not specifically point out the role of these structures as being either a cause or a consequence of the disease. Genetic studies can also be performed with these post-mortem tissues to identify a potential mutation as the cause of the disease, such as in HD.

In ALS, 20% of the patients have at least another family member affected by the disease, even though only approximately 10% of these individuals possess a SOD-1 mutation, the most well-characterized ALS mutation so far. As for the sporadic cases of ALS, a combination of environmental factors with potential genetic susceptibility, in addition to other causes such as microtraumas, are currently being suspected.

The use of animal models that mimic the diseases can be of great help, but a minimal understanding of the etiology of the diseases need to be reached before those models can be generated. PD can be induced in rats following destruction of their substantia nigra, and ALS transgenic mouse model can be generated by overexpression of the human mutated SOD-1 gene. However, studying the effects of the disease on an entire animal remains complex and does not always bring a clear answer to the questions raised. In ALS for example, it is still not well understood how the SOD-1 mutation induces motor neuron degeneration, even though many research groups worldwide have been working on the transgenic mouse model for more than a decade now.

Thus, in vitro culture systems could be very helpful to perform dynamic studies at the cellular level. However, culturing neurons faces major limitations, because neurons are nearly impossible to extract from the adult brain or spinal cord, and these cells do not proliferate in vitro. This is why neurons are usually extracted from mouse or rat embryos at an early stage of development (E12–E14), before they have had the time to establish too many connections. In addition, since neurons do not proliferate, cell extraction from embryos needs to be performed again for each experiment.

Finally, a broad limitation for these cultures, in common with most of the other cell types, consists of the vast difference in environment that
occurs between a cell cultured on a plastic dish in two-dimensions, compared to the three-dimensional environment in situ. Both the cell-cell and cell-matrix contacts are totally different, as well as the global cell behavior, especially for the nervous system in which multiple connections between neurons are constantly established.

3.1 Two-dimensional in vitro models of neural cell culture

In vitro cultures of various types of neurons (hippocampal, motor, sympathetic, etc.) have been performed for several years now, by extracting cells from the nervous system of fetuses. These culture systems allow for the isolation of purified neurons from their surrounding environment, which greatly facilitates studies such as mRNA or protein analysis. However, it has been noted that neurons usually only survive over a short period of time in the absence of glial cells.

Mixed culture of spinal cord cells can be maintained for an extended period of time, and neurons can be microinjected with a plasmid to over-express a protein of interest (mutant or not) to study its impact on cell physiology or survival.\textsuperscript{15}

Double compartment cultures have been developed by using neurons cultured on a plastic dish, and glial cells attached to a glass slide and then flipped over and put onto the neurons using spacers so that cells would be as close as possible from each other for studying the paracrine effects occurring without direct cell-cell contact.\textsuperscript{16}

Some in vitro models have also been developed to mimic a traumatic injury, through the stretching of cortical neurons cultured on elastic substrates and the subsequent analysis of their intracellular calcium concentration.\textsuperscript{17}

3.2 Three-dimensional tissue-engineered models of the nervous system

Spinal cord or brain tissue slices can be maintained as organotypic cultures in vitro, preserving the three-dimensional organization of the nervous tissue with all of its cell-cell contacts. These models are very useful as they are highly physiological, allowing electrophysiological studies to be performed only a few hours following the tissue harvesting.\textsuperscript{18}
However, they do not provide any sort of control on the cell types present in the tissue of interest, and the specific cells’ proportion and condition.

What could be really helpful to study neurodegenerative diseases would be to reconstruct the nervous system by independently combining each cell type. This will be particularly useful with the multiple transgenic mouse models developed to mimic various disorders, such as the overexpression of the human mutant SOD-1 gene in the G93A ALS mouse model. It would be even more interesting if we could be using the patient’s own stem cells differentiated into specific types of neurons and glial cells, providing that these cells would keep their diseased phenotypes over the full course of differentiation.

Some three-dimensional neural constructs were developed by coculturing neurons with astrocytes in a 500–800 μm thick 3D Matrigel™. Cells within these constructs displayed extensive 3D process outgrowths and a high viability over multiple weeks. In addition, neurons in this model can be tested through patch-clamp techniques, and were shown to be able to display electrophysiological action potentials and functional synapse formation.19

However, a drawback with the use of Matrigel™ is that it consists of a material containing multiple active molecules, such as laminin, collagen IV, entactin, heparan sulphate and cytokines, in undetermined concentrations.

Other biological polymers characterized by better controlled compositions, such as collagen, fibrin, methylcellulose or agarose, have also been used as culture scaffolds to reconstruct 3D neural tissues.19

We developed a tissue-engineered model of motor neuron culture to study the axonal migration and myelination processes in a three-dimensional environment. Since most of the motor neuron’s cell surface is located outside of the spinal cord, coupled with an axon that can be measured up to one meter long, the process of axonal migration represents a major issue in the study of motor neurons. Moreover, this axon is also being myelinated by Schwann cells, the main glial cell type of the peripheral nervous system.

To closely mimic the 3D environment surrounding motor neurons, this peripheral nerve migration aspect should be taken into account. To recapitulate the tissues through which nerves make their way to the muscle, we developed a 3D connective tissue made of fibroblasts cultured in a
collagen sponge and maturated for two weeks in order to promote extracellular matrix deposition. Mouse motor neurons extracted from E12 mouse embryos were purified through density gradient centrifugation, and then seeded on top of the reconstructed connective tissue (Fig. 1). The motor neurons formed a thick and dense cell layer over it. To promote axonal migration, the sponge was lifted at the air-liquid interface, and a

Fig. 1. Preparation of a tissue-engineered model to study axonal migration and myelination of motor neurons through a 3D connective tissue.
cocktail of neurotrophic factors was added to the culture medium underneath. As shown by immunohistochemistry, a large number of neurofilament M-positive neurites was observed migrating down from the neurons layer to the bottom of the connective tissue for over up to 1 mm long in distance. In addition, when mouse Schwann cells were co-cultured with the fibroblasts in the sponge, they migrated alongside with the neurites, as shown by the Myelin Basic Protein double-staining with Neurofilament-M. An analysis of these neurites, performed by transmission electron microscopy, showed that a thick myelin sheath was wrapped around some of the neurites after 28 days of in vitro maturation (Fig. 2). Thus, this tissue-engineered model was shown to promote axonal migration, axon myelination by Schwann cells, and spontaneous myelin sheath formation for the first time in vitro.

This model should greatly facilitate studies on diseases related to motor neuron axon demyelination, such as MS or Charcot-Marie-Tooth disease of the peripheral nerves.

In addition, it could be very valuable to study ALS with such a model, by using different combinations of spinal cord cells obtained from SOD-1 mutant mice versus wild-type mice, all the while measuring motor neuron degeneration.

Finally, this model could be even more interesting if it could be reconstructed using the patient’s own cells, to mimic the human disease in vitro. Since living motor neurons cannot be extracted from an adult spinal cord, i.e. from post-mortem tissues, other alternatives should be explored in order to generate these cells from the patients. One of the best solution appears to proceed with the differentiation of motor neurons from adult stem cells.

In addition, the differentiation of neurons from autologous stem cells could be applied for cell replacement therapy applications, as a novel approach to treat neurodegenerative disorders by implantation of new and functional neurons.

4. Neural Stem Cells to Treat Neurodegenerative Diseases

After molecular biology, gene therapy, nanotechnology and tissue engineering, stem cells are now considered as representing the new
Fig. 2. (A) Fibroblasts and Schwann cells were co-cultured for 21 days in the collagen sponge, and were observed by histology staining with Masson’s trichrome. (B) Mouse motor neurons were seeded on top of the sponge and cultured for an additional 14 days. (C) The neurons layer (stained with an antibody against Neurofilament-M) was cultured at the air-liquid interface, but without the addition of neurotrophic factors; neurites did not migrate through the sponge. (D) The culture medium underneath the sponge was supplemented with neurotrophic factors, which promoted neurite migration.
emerging field that could revolutionize the future of medicine. This is particularly true in neurosciences, field in which the work with human neurons is still impracticable, and for which the use of neural cells differentiated from human stem cells may be the new standard in a near future. To fully understand the current known potential and limitations of stem cells, as well as their diversity and specific related issues, a rapid overview of fetal neurons, as well as a comparative standpoint of embryonic versus adult stem cells, will be presented.

4.1 Human fetal neural transplantations, a proof of concept for cell replacement

Even though cell transplantation procedures have already been in clinical practice for numerous organs, attempts at replacing cells within the CNS do remain experimental. PD readily represents a candidate disease of choice for cell replacement therapy since the vast majority of its lost dopaminergic neurons come from a circumscribed area, the substantia nigra compacta. This specificity made it the first disease to be considered and treated by cellular therapy. The first attempt was made using heterologous fetal neurons obtained from aborted fetuses, mainly because only fetal neurons can survive tissue extraction. This initial series of transplantation studies provided us with insights suggesting that the grafted cells could, at least partially, survive, integrate the brain, and improve the disease’s symptoms for an extended period of time.

4.1.1 Parkinson’s disease

It was during the late 1980s that the very first methodical clinical transplantation trials using fetal dopaminergic neurons in patients with PD were elaborated. Mesencephalic tissue (an area in the developing CNS rich in immature dopaminergic neurons) of six to nine weeks old aborted fetuses

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Fig. 2. (Continued) through the connective tissue. (E) A transmission electron microscopic picture of the sponge showed that some neurites were wrapped with thick myelin sheaths, observed with a higher magnification in (F) Bar in B, 60 μm for A–B; Bar in D, 100 μm for C–D; Bar in E, 2 μm; Bar in F, 0.2 μm. (Modified from Ref. 58.)
was transplanted into the striatum. Various immunosuppression policies were adopted, ranging from none, to cyclosporin A for six months, and to a cocktail mix of cyclosporin A, steroids, and azathioprine.

Of the open-labeled trials, without the presence of a control placebo group or blinding procedures, the initial results following fetal nigral grafts in PD patients showed graft survival and clinical improvements in these PD patients. From that timeframe, over 400 PD patients throughout the world have been grafted with nigral tissue, and improvements were reported from none to long-lasting dramatic benefits.

Of the double-blind placebo controlled trials, in which there was presence of a sham group consisting of the PD patients being anesthetized, their skull perforated but without the actual cell transplantation step occurring, both the patients and observers remained blinded to this condition. In one study, 19/40 patients received a tissue strand cell transplantation, after which a subjective global rating scale test was conducted one year post-transplantation.

Slight improvement was noted in the transplanted group in comparison to the sham one, but 15% of the patients ended up with severe off-dyskinesias. In another study, solid pieces of embryonic ventral mesencephalon have been transplanted into the putamen of both sides of the brain. Eleven patients were sham controls. After two years, no net improvement was noted, and 56% of the patients resulted with off-dyskinesias.

No real positive effect using cell transplantation was detected in those studies. Possible reasons for this poor outcome may be that the clinical assessments were not standardized accordingly, or the tissue preparation, long-term in vitro storage, immunosuppression use, and surgical approaches had design flaws.

Interestingly, analysis of post-mortem tissues more than ten years after graft of fetal neurons showed that, whereas some of these cells underwent pathological changes similar to Parkinson disease (formation of Lewy bodies), the majority of them did not display evidence of functional impairment or degeneration.

4.1.2 Huntington’s disease

Fetal neural transplants have also been used to treat patients suffering from HD. A recent study about fetal neural transplants reports cases of three HD
patients autopsies, performed ten years post-transplantation. In two out of those three patients, there seems to have been a differentiation into adequate cell types and proper glutamatergic and dopaminergic innervating projections from the recipients’ brain. Yet, degeneration was observed in the grafted cells. The grafts also showed astrogliosis, inflammatory infiltrates, and microglial activation. Given that only temporary and minor benefits, along with considerable graft degeneration were observed, this would limit the use of fetal cells for eventual studies conducted for HD.

Human fetal neural transplantations applied to hundreds of PD patients over the last two decades have set new standards for cell transplantation into the brain. However, the risks linked with human fetal transplantation trials may very well be higher than initially expected. In fact, there are obvious logistic problems linked with the amount of donors needed for each patient, on top of all of the ethical concerns that have been raised concerning the use of cells obtained from aborted fetuses. Put together, these factors are widely restricting the application of fetal tissue for neural transplantation. Nevertheless, even if this cell replacement therapy could be proven successful in only a few cases, by promoting cell survival and integration into the neuronal circuitry and, ultimately, for long-term benefits for the disease’s symptoms, it still shows that cell therapy applications could be successful, once a better control of the graft parameters will have been established.

4.2 Embryonic stem cells (hESC)-derived neural precursor cells

Fetal neural transplantations are limited, not only by the availability of fetuses but also largely by ethical concerns. One alternative could be the development of pluripotent stem cell banks generated from embryos at an earlier developmental stage (supernumeraries not used during in vitro fertilization), which may be not as ethically controversial as fetuses, and which could generate large amounts of various cell types due to their high proliferative and differentiation potentials (Table 1).

The use of hESC-derived NPCs led to some promising results in the treatment of PD and HD. However, concerns similar to those related to fetal neural transplants have been raised for those cells: the hESC-derived NPCs are heterologous and thus could be targeted by the host immune
system, while also facing major limiting ethical concerns. Meanwhile, hESCs undoubtedly hold the highest proliferative potential and the largest and most efficient differentiation capacity in comparison to adult NPCs.

### 4.3 Adult tissue-derived neural precursor cells (NPCs)

The idea of growing cells in culture is particularly attractive if the cells consist of adult tissue-derived NPCs, able to differentiate into various neural cell types. Studies are currently being done to develop methods aiming at modifying adult stem cells into precursor cells, opening up the possibility to harvest a patient’s own cells and rendering them suitable for transplantation into the nervous system (Table 1).

#### 4.3.1 Brain-derived NPCs

NPCs were initially defined as the self-renewing, multipotent cells that generate the main phenotypes of the nervous system, of both neuronal and glial subpopulations. Ever since the first subpopulation of mitotic NPCs was identified in the adult mice brain tissue,$^{34}$ NPCs have been isolated from various species including human.$^{35}$

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<td>Pluripotency (extended potential)</td>
<td>Multipotency</td>
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<td>Multilineage differentiation</td>
<td>Limited risk of tumor formation</td>
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<td>Extensive self-renewal</td>
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<td>Access to early neural development stage</td>
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<td>Ease for inducing stable genetic changes</td>
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<td>Risk of immune rejection by the host</td>
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| Table 1. Comparison of Human ESCs vs. Adult NPCs. |
The maintenance of NPCs is ensured by the NPC niche, in which microenvironmental cues, but also interactions implicating the extracellular matrix and the cellular membranes, cell–cell interactions and the proximity to blood vessels, aid in maintaining cell proliferation, fate specification and differentiation.\textsuperscript{36}

In the past 15 years, research groups have put a lot of efforts onto NPCs derived from various regions of the developing brain in order to stimulate the promotion of the functional recuperation process by dopaminergic differentiation in PD rat animal models.\textsuperscript{37} Even if characteristics such as graft survival, neuronal and astrocytic differentiation, cell migration, and axonal extension have been demonstrated, these types of studies had not yet shown evidence that a significant dopaminergic differentiation had set place.\textsuperscript{38} However, given the actual location of these NPCs within the brain, some limitations still prevent their use for applications in cellular therapy.

4.3.2 Bone marrow-derived NPCs

Other cell types generated from non-brain derived tissues, such as bone marrow, have been approached, having been shown to express, for example, certain dopaminergic markers. Mesenchymal stem cells (MSC) obtained from adult rodent have been shown to differentiate, both \textit{in vitro} and \textit{in vivo}, into cells with mesenchymal, visceral mesoderm, neuroectoderm, and endoderm characteristics.\textsuperscript{39} A subtype of these MSC was isolated and termed multipotent adult progenitor cells (MAPC). Surprisingly, it has been reported that as many as 30\% of mouse MAPCs differentiated \textit{in vitro} into tyrosine hydroxylase (TH, the rate-limiting enzyme in the production of dopamine)-expressing neurons. Those cells were presupposedly pluripotent, opening the possibility for MSC to be a cell source of choice for neurotransplantation in PD. Human bone marrow stromal cells were also shown to be able to convert into a NPC-like population that allowed for the expression of neuronal markers once differentiated, of which about 11\% of the cells were TH-positive and were shown to release dopamine upon membrane depolarization.\textsuperscript{40} However, the functionality of those cells remains to be shown, and so additional studies in PD animal models are still necessary.
4.3.3 Skin-derived and adipose-derived NPCs

It has previously been shown that differentiation of NPCs isolated from human adult skin could generate neural (and mesodermal) derivatives, while potentially providing a readily accessible source of human adult NPCs for transplantation: the skin-derived progenitor or precursor cells (SKP). These cells possess distinct surface markers in common with MSCs, while preferentially differentiating into neural cell types. It appears that these SKPs can be passaged for up to one year without showing any significant senescence and seem to behave similarly to NPCs extracted from the brain in the way that they have the ability to form floating spherical colonies called neurospheres (Fig. 3). The achievability of expansion and possibility for long-term culturing of SKPs, coupled with their versatility, give them a certain appeal for use in therapy for disorders of the nervous system. SKPs have also been shown to generate Schwann cells. The differentiated Schwann cells were transplanted into rat spinal cords following an induced traumatic injury, and appeared to survive within the injured environment and to myelinate host axons, all the while improving locomotor recovery.

Adipose tissue has been identified in 2001 as an alternative source of multipotent stromal MSCs, which can be obtained by a less invasive method and in larger quantities compared with skin or bone marrow NPCs (through liposuction). These cells can also be a source of NPCs. The ability of Adipose-Derived Adult Stromal (ADAS) to neuroprotect or restore function in an injured dopaminergic pathway was investigated after transplantation of naive or neurally-induced ADAS into the striatum of parkinsonian rats. ADAS failed to generate stable dopaminergic neurons in situ, but gene expression analyses showed that both naive and differentiated ADAS cells express neuroprotective and trophic factors at the lesion site.

4.3.4 Induced pluripotent stem (iPS) cells derived-NPCs

In recent years, several approaches have been developed in order to reprogram differentiated adult cells into pluripotent stem cells, named induced pluripotent stem (iPS) cells. Somatic cells have first been reprogrammed
Fig. 3. (A) Neurospheres-forming human skin-derived NPCs (phase contrast microscopy). (B) Human skin-derived NPCs differentiated in Neurofilament-M-expressing neurons (immunohistochemistry). (C) Human skin-derived NPCs differentiated in TH-expressing neurons. Nuclei were stained with Hoechst. Bar in A, 200 μm; Bar in C, 25 μm for B–C.
into iPS by viral transduction of four transcription factors, c-Myc, Oct4, Sox-2 and Klf4$^{51,52}$ and then by the expression of only Oct-4 and either Klf-4 or c-Myc.$^{53}$ More recently, it has been shown that the generation of adult iPS cells was achievable by no means of genetic modifications.$^{54}$

A study has shown that iPS cells dedifferentiated from skin fibroblasts could undergo a differentiation into NPCs, then into dopaminergic neurons. After transplantation into the brains of parkinsonian rats, the grafted cells showed neuronal extensions throughout the neighboring brain regions, all the while lessening the PD-like symptoms.$^{55}$

Autologous transplants using a patient’s own cells are now at hands reach for the potential development of human adult NPC-based therapies for neurodegenerative diseases. It would now be possible to generate pure and highly proliferative colonies of iPS cells that, in theory, would have an equivalent potential as embryonic NPCs, additionally to being autologous, non-ethically restricted cells. But these avenues also come with a major setback: they also hoist the likelihood to induce tumor formation. Taking that iPS cells may be proven to be just as or even more performing than human adult NPCs, and all along being easier to extract, culture and control, they may very well become a cell type of choice for the treatment of neurodegenerative disorders.

### 4.4 Advantages and limitations of NPC culture

The main advantage with the use of human tissue-derived NPCs consists of the possibility to isolate cells from an autologous tissue, whether it be skin, fat or any other somatic tissues for iPS cells. Patients can give their informed consent, hence circumventing the ethical concern. Harvesting skin, fat tissue or even bone marrow can be done quickly, through a minimally painful procedure with local anesthesia. The next steps are more challenging. First, NPC purification remains a major issue since no cell surface antigen allowing for immunoprecipitation enrichment has been identified so far, but some interesting purification steps can readily be achieved through floating neurospheres selection (Fig. 3).$^{47}$

Another limiting step that still remains is the NPC proliferation. Since the amount of autologous tissue that can be harvested is limited, the proliferation step is crucial to generate enough NPCs for transplantation
purposes. The steps for deriving and expanding human adult NPCs are difficult to standardize, extremely complex and expensive to perform, and have not been really efficient.

Also, once NPCs have been grown, they need to be differentiated into the adequate subtype of neurons, depending on the neurodegenerative disorder that needs to be addressed. In that regard, the differentiation potential of human adult NPCs seems to still be more restricted than that of hESCs.56

Overall, human adult NPCs appear to be more complicated to culture and consequently it is more challenging to control their fate while trying to generate specific types of neurons.57 One of the next major challenges would consist of generating enough human adult NPCs to use in therapeutic replacement of damaged tissues in the nervous system. However, issues such as cell survival, appropriate synaptic integration into the host brain, behavioral recovery, and tumor formation need to be addressed. Nevertheless, these limitations may be compensated by the autologous nature of these cells, and the absence of ethical concern.

5. Conclusion

Human adult NPCs now represent a true possibility and a open door to several other studies to come on not only cell transplantation as a therapeutic approach for various disorders of the nervous system, but also on the creation by tissue engineering of 3D models to study the diseases.21 The combination of these models with the use of human adult neuronal and glial cells generated from the differentiation of NPCs isolated directly from the patients and from readily accessible tissues sources (skin, fat, bone marrow) or iPS cells, will enable the development of powerful models to better understand human neurodegenerative diseases. Lastly, with the use of autologous human adult NPCs, therapeutic transplants may become one of the next best long-term hopes for reversing neurodegenerative diseases.

Acknowledgments

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References


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1. Introduction

Stem cells have the unique ability to grow indefinitely (proliferation) or adopt new cellular fates (differentiation). As such, stem cells represent a unique cell-based system to study and model human development and diseases, to screen safety and mode of action of novel drugs and to provide the raw material for cell-based therapies of presently incurable diseases. Human pluripotent stem cells (hPSCs), which are able to differentiate into all cell types in the human body, have the potential to revolutionize the treatment of many human disorders for which no effective therapies presently exist. Experimental manipulation of these cells to affect proliferation and differentiation is central to developing strategies for the production of defined and mature cell types that can be used to study development and disease progression, to perform drug screens, and to treat a variety of degenerative disorders such as Alzheimer’s and heart disease.

Conventional cell culture methods are limited in their ability to screen the vast number of factors that can influence stem cell behavior. The
establishment of high-throughput screening (HTS) technologies with stem cells is important for a broad range of applications from basic understanding of the role of certain signaling networks in self-renewal to the development of novel therapeutic approaches, such as cell replacement of damaged, diseased or dead tissues. HTS technologies generally consist of three interrelated components: (1) platform fabrication, (2) data acquisition and (3) data analysis and mining (Fig. 1). The behavior of stem cells can be controlled either by altering their extracellular environment (extrinsic manipulation) or by interfering with intracellular signaling pathways and transcriptional networks (intrinsic manipulation). This chapter discusses the emerging trend of using HTS technologies for the extrinsic and intrinsic manipulation and engineering of stem cells.

2. Sources of Stem Cells Suitable for High-Throughput Screening Approaches

A stem cell is defined by its ability to (1) maintain its undifferentiated, or “blank slate,” state and (2) differentiate into mature and specialized cell types. Broadly speaking, there are two types of stem cell populations, which vary significantly in their properties. Pluripotent stem cells can in principle be maintained indefinitely in an undifferentiated and highly proliferative state while retaining their pluripotency (i.e. the ability to differentiate into all mature cell types). In contrast, adult stem cells are restricted in their potency (multi- or uni-potent), giving rise to only a...
subset of specialized cell types. In addition, adult stem cells are extremely rare and hence difficult to isolate in pure form and expand in an undifferentiated state in the culture dish. Given these limitations, high-throughput screens are often not feasible with adult stem cells, and pluripotent stem cell lines provide a powerful alternative.

Until recently, human pluripotent stem cells were mainly derived by dissection of the inner cell mass from the blastocysts. The discoveries by Takahashi and Yamanaka ushered in a new era for human pluripotent stem cells: by introducing a combination of transcription factors into mature and specialized cell types, it is now possible to derive pluripotent stem cells, or induced pluripotent stem cells (iPSCs), that are nearly identical to embryonic stem cells (ESCs). This technology permits the derivation of patient-specific stem cell lines. Together, embryonic and induced pluripotent stem cell lines provide a virtually limitless supply of human cells for large-scale high-throughput screens of any kind.

3. The Stem Cell Niche: A Cellular Microenvironment That Controls Stem Cell Behavior

The in vivo cellular microenvironment is a complex mixture consisting of four distinct “components”: (1) Immobilized protein factors such as extracellular matrix proteins (ECMPs) interact with the cell through integrin binding. (2) Soluble protein factors (such as growth factors, small molecules, and hormones) influence cell signaling pathways via their appropriate extracellular receptors. (3) Mechanical forcers (such as stretch or shear stress) modulate intracellular signaling processes by activating mechanoreceptors. (4) Neighboring cell types communicate with each other by cadherin-mediated signaling (Fig. 2). Each of these four components include tens to thousands of “members” that influence numerous signaling pathways, which perturb gene and protein expressions, and ultimately affect cell fate and cell function.

Members of each of these components interact in complex manner to influence each other’s signaling ability, a phenomenon known as crosstalk. For example, endothelial cell attachment to fibronectin via $\alpha_5\beta_1$ integrin potentiates $\alpha_v\beta_3$-mediated migration on vitronectin. Along similar lines, growth factors and ECMPs often have a reciprocal relationship — cell
adhesion to ECMPs is required for activation of growth factor receptors, and growth factors are necessary to stimulate cell adhesion, migration, and the resulting integrin-dependent response. These interactions, which can be synergistic or antagonistic, are well documented in numerous biological systems.\(^4,5\) For example, \(\alpha\nu\beta3\) integrin associates with activated insulin growth factor (IGF) receptor and platelet derived growth factor (PDGF) receptor, and potentiates the biological activity of IGF and PDGF, respectively.\(^6\) Likewise, in human blood mononuclear cells, collagen-induced release of interleukin 1 (IL-1) through the binding of integrin \(\alpha7\beta1\) is potentiated by fibronectin binding to \(\alpha5\beta1\).\(^7\) These examples demonstrate the importance in crosstalk in regulating cell fate. Since these interactions are complex and not predictable from studies on individual members of the various components, high-throughput systematic approaches to study crosstalk in stem cells are needed.

Fig. 2. Cellular microenvironment. Four distinct components of the cellular microenvironment that affect cell fate. (1) Immobilized proteins such as extracellular matrix proteins interact with the cell through most commonly through integrin signaling. (2) Cell-to-cell interactions are mediated through cadherin signaling. (3) Chemical stimuli such as growth factors and hormones interact with the cell through their respective receptors. (4) Mechanical stimuli such as shear stress or flow signal the cell through mechanoreceptors. These distinct compartments have numerous members that interact in a complex manner, known as crosstalk, to affect a variety of signaling pathways which in turn affect gene and protein expression and ultimately cell fate and function.
In vivo, stem cells reside in specialized microenvironments of organs and tissues called niches that regulate self-renewal and differentiation. The niche also serves to balance the choice of a stem cell to self-renew or differentiate: excessive self-renewal could lead to cancer, while differentiation could lead to depletion of a tissue’s regenerative potential. Thus, the niche must maintain a delicate balance between stem cell proliferation and differentiation.

Stem cell niches of various tissues with regenerative potential, such as skin, stomach, intestine and blood, share certain structural and organizational properties. The niche cells provide cell-cell contacts and paracrine signaling that regulate the self-renewal of the neighboring stem cells. The ECM provides a scaffold for stem cell growth in the niche and can interact with soluble factors to regulate signal transduction. Additionally, glycoaminoglycans serve to locally concentrate and present soluble cytokines. The physiochemical environment, including oxygen gradients, pH, matrix stiffness, and topography, also contribute to the regulation of stem cell proliferation, self-renewal, and differentiation.

The first stem cell niche identified in mammals was the hematopoietic stem cell (HSC) niche. Individual HSCs are multipotent and highly self-renewing, but yet proliferate quite slowly. However, the manner in which HSCs interact with their niche to promote self-renewal has not been elucidated. Furthermore, the few existing culture systems that allow for maintenance and expansion of HSCs in vitro are not well defined. More recently, niches have been identified in a wide range of tissues such as the skin, brain, gut, and liver. Another well-characterized stem cell niche is that of the intestinal stem cells. Intestinal stem cells can be isolated to near homogeneity using the marker gene Lgr5, and these cells produce in vitro an intestinal-like structure that generates its own niche cells to maintain the Lgr5-marked stem cells. However, in most cases, expansion of stem/progenitor cell populations in vitro without loss of stem cell potential is often difficult or even impossible.

HESCs and iPSCs are able to generate all derivatives of the three primary germ layers — ectoderm, endoderm and mesoderm — a property referred to as pluripotency. Unlike tissue-specific adult stem cells, hESCs are only transiently present during development and do not have a stable niche in vivo. Nonetheless, various in vitro culture conditions for
hESC proliferation and differentiation have been developed. A combination of components, including ECMPs, soluble factors, and other physiochemical factors act to maintain and expand the stem cell population or promote particular differentiation programs. However, given the myriad of factors that may influence hESC proliferation and differentiation, the in vitro culture conditions and the cellular microenvironments that either promote hESC expansion or their specific differentiation have not been successfully developed and defined. Thus, the development of HTS methodologies is necessary in order to identify culture conditions for the maintenance, expansion, and directed differentiation of stem cells.

### 3.1 Traditional HTS for identifying modulators of stem cell fate

Traditional HTS uses microtiter plates along with robotics, liquid handling devices, and automated imagers to conduct rapidly thousands to millions of conditions (Fig. 3A). A highly sensitive, robust and quick read-out is critical, as is the case for any HTS method. Most HTS typically involve the use of libraries consisting of hundreds of thousands or even millions of small molecules that are created through combinatorial chemistry approaches, in order to identify compounds that produce the desired phenotype.\(^{17,18}\) These screens are a useful tool to address questions in basic stem cell biology and chemistry.

Many groups have used HTS of chemical libraries to identify small molecules that regulate mouse ESC (mESC) self-renewal. For example, a transgenic reporter mESC line that expresses GFP under control of Oct4, a marker of pluripotency, was utilized to screen 50,000 compounds under differentiation conditions free of leukemia inhibitory factor (LIF) and feeder cells. This high-throughput cell-based screening approach was used to identify several small molecules that promote the self-renewal of mESCs in the absence LIF and feeder cells. Specifically, these authors identified a previously uncharacterized heterocycle, SC1/pluripotin, that allowed for the propagation of mESCs in an undifferentiated, pluripotent state in the absence of feeder cells, serum and LIF.\(^ {19}\)

These traditional HTS technologies have also been used to identify various small molecules that promote mESC differentiation into multiple
lineages. A high-throughput phenotypic cell-based screen of kinase directed libraries led to the identification of a synthetic small molecule inhibitor of glycogen synthase kinase-3β (GSK-3β), TWS119, that induces neurogenesis in mESCs.\textsuperscript{20} Previously, a distinct GSK-3β inhibitor, called BIO, was shown to promote the undifferentiated growth of ESCs. Likewise, using a combinatorial library of peroxisome proliferator-activated receptor (PPAR) ligands, novel molecules that promoted mesodermal differentiation of murine ESCs into beating cardiomyocytes were identified.\textsuperscript{21} Along similar lines, a phenotypic cell-based screen of a large combinatorial chemical library was utilized to discover a class of diaminopyrimidine compounds that efficiently induce mESCs to differentiate into cardiomyocytes.\textsuperscript{22}
The development of HTS for hESCs has been difficult because of challenges involved in establishing suitable growth conditions. More recently, though, a strategy was developed to adapt hESCs to HTS conditions, and to screen 2880 compounds for their effects on hESC self-renewal or differentiation (Figs. 3B and 3C).23 Use of this HTS system resulted in the identification of several drugs and natural compounds that promote short-term hESC maintenance and compounds that direct differentiation.23 A similar approach was used to identify a small molecule, stauprimide, that down regulates c-Myc and thus increases the efficiency of the directed differentiation of hESCs.24

3.2 Cellular microarray-based screening in stem cell research

Although HTS have greatly advanced modern biology and drug discovery, they are not practical for all stem cell related studies given the cost and the large number of cells and reagents required. For example, typical HTS require $25–50 \times 10^4$ cells per condition screened.23 Thus, HTS are not feasible for screens involving adult stem cells, which are rare (e.g. one in 200,000 blood cells is a hematopoietic stem cell) and difficult to isolate. HTS using conventional multi-well plates is cost-prohibitive and often does not provide adequate quantitative information on cell function. Additionally, most HTS approaches only have the capacity to investigate the effects of one factor at a time, often ignoring the complex crosstalks that typically occurs in biological settings between combinations of molecules.

In order to overcome these obstacles, cellular microarrays have been used for screening the effects of large numbers of biological molecules on stem cell fate.25 Typical cellular microarrays consist of a chip (e.g. glass microscope slide) where minute volumes ($\mu$L to nL) of various molecules (e.g. ECMs, small molecules, cytokines, biopolymers) are deposited in defined locations and analyzed for their effect on cellular processes (e.g. changes in gene and protein expression levels). These arrays are fabricated using robotic spotting, photo-assisted, and soft-lithography approaches.
A major challenge in developing stem cell-based therapies is the identification of conditions that specifically regulate and influence their fate. Being able to identify components that mimic the stem cell niche will aid in the expansion and differentiation of stem cells in vitro. Considering the complexity of the microenvironments in which stem cells reside, it is impractical to test proliferation and differentiation conditions using current established HTS methods. Cellular microarrays have advantages over traditional well-based HTS in that they provide more information from smaller sample volumes in a rapid, efficient, and cost-effective manner.

3.2.1 Combinatorial protein arrays for studying stem cell microenvironments

Combinatorial protein arrays consist of immobilized biological signaling molecules on a surface onto which cells are seeded (Fig. 4). The first such platform was used to screen ECMPs and their effects on stem cells in a combinatorial fashion. Specifically, with the use of a DNA robotic spotter, different ECMP combinations were spotted, and the commitment of mESCs towards an early hepatic fate was evaluated. Several combinations of ECMP components that influenced stem cell differentiation and hepatocyte function were identified. This platform was further developed to investigate the interactions between ECMPs and soluble growth factors on stem cell fate. Several ECMPs and growth factors were found to influence mESC differentiation towards the cardiac lineage.

Similar robotic spotting techniques were used to print arrays of ECMPs along with growth factors and adhesion molecules to evaluate their effects on the proliferation and differentiation of human adult neural precursor cells, and the results revealed significant effects of certain signaling molecules (such as BMPs, Wnts, and Notch) on the extent and direction of differentiation into neuronal or glial fate. For example, it was found that Wnt and Notch co-stimulation maintained the cells in an undifferentiated state. Along similar lines, a photo-assisted patterning process was used to create matrix-growth factor arrays to identify materials that direct neural stem cell growth and differentiation.
Fig. 4. Combinatorial protein array technology for manipulating hESC fate. (A) Typical layout of arrayed cellular microenvironments. Arrays of pre-mixed combinations of extracellular matrix proteins (ECMPs) and signaling molecules are printed onto acrylamide coated glass slides using a contact microarray printer. Human embryonic stem cells (hESCs) were cultured on the arrays for 5 days. Cells were imaged live (B–C) and then fixed and stained for DNA (D–E) and Nanog (F–G) to identify conditions that hESC attachment, proliferation, maintenance of pluripotency and differentiation. (Figure and legend adapted from Ref. 30 with permission.)
Although these platforms were used to elucidate the role of certain microenvironmental components on stem cell fate, they have limitations. Specifically, these platforms either relied on the addition of signaling molecules to the surrounding media, thereby limiting the throughput and the complexity of the microenvironments that could be screened, or involved the covalent attachment of the signaling molecules to the chip, thus affecting their biological activity. Additionally, the throughput of these systems (<100 conditions per chip) was significantly lower when compared to conventional HTS systems. In order to overcome these limitations, an integrated array platform was developed in which ECMPs, growth factors, and small molecules were non-covalently arrayed on acrylamide-coated slides, thereby creating comprehensive microenvironments that closely resemble the *in vivo* microenvironment in which cells reside (Fig. 4). This technology platform was used for the real-time simultaneous screening of thousands of physiochemical parameters on stem cell attachment, proliferation, differentiation and gene expressions. Specifically, through the systematic screening of ECMPs and other signaling molecules, a completely defined culture system for the long-term self-renewal of three independent hESC lines was developed. In another study, this technology was used to investigate the effects of microenvironmental modulations on the fate of hepatic stellate cells (HeSCs), a progenitor cell that resides in the liver. It was determined that different components of the microenvironment differentially influence other components to influence the HeSCs phenotype. For example, it was found that the influences of Wnt signaling molecules on HeSC fate are dependent on the ECMP composition in which they are presented. Furthermore, this array platform technology was validated by the finding that data obtained from these experiments were indistinguishable from data obtained from traditional multi-well-based assays.

### 3.2.2 Polymer arrays for screening of biomaterials that influence stem cell fate

Biomaterials have been used for the expansion of many human adult stem cell and progenitor populations. Mesenchymal stem cells (MSCs), for example, were maintained and differentiated on several different classes...
of synthetic and natural biomaterials.\textsuperscript{32–36} Unmodified and methyl-modified silane surfaces were used to enhance MSC proliferation.\textsuperscript{32} Likewise, neural stem cells (NSCs) were maintained and expanded on 3D scaffolds composed of amino polymers such as poly(D-lysine).\textsuperscript{37} There has been some progress in the application of polymers for expansion and maintenance of ESCs. For example, aliphatic poly(\(\alpha\)-hydroxy esters) such as poly(D,L-lactide) and poly(glycolide) were used to propagate mESCs.\textsuperscript{38} Nonetheless, biomaterial-based expansion and differentiation of stem cells has been slow coming. This has been mainly due to the inefficiency of the iterative nature of biomaterials-based research in which materials are fabricated, tested, and redesigned.\textsuperscript{39}

In order to establish a set of principles or properties that can assist in prediction of which polymers would influence stem cell fate, high-throughput array-based approaches have been implemented. In one study, an array-based biomaterials screen was used to identify specific functional groups that promote human MSC (hMSC) differentiation,\textsuperscript{40} it was found that phosphate surfaces promoted osteoblast formation, while t-butyl-modified surfaces promoted adipocyte formation. A similar approach was implemented to study the effects of 576 synthetic materials on stem cell differentiation (Fig. 5).\textsuperscript{41} Using this method, several classes of polymers that promoted high levels of differentiation into epithelial cells were identified. More recently, this technology has been used to identify biodegradable polymers that support the growth and expansion of hMSCs and neural stem cells.\textsuperscript{39}

3.2.3 Microwell approaches to analyze physical cues regulating stem cell fate

By using soft lithography methods, microwell arrays with defined dimensions can be fabricated (Fig. 6). For example, microfabrication was used to create an array of approximately 10,000 microwells on a glass coverslip for the parallel, quantitative analysis of single ESCs.\textsuperscript{42} Furthermore, the well dimensions could be adjusted over ranges of 10–500 \(\mu\)m in height and 20–500 \(\mu\)m in diameter, and the platform was compatible with traditional light and fluorescent microscopy. The microwell platform was used to investigate the effect of cell density on the proliferation dynamics of rat neural stem cells (NSCs).
Fig. 5. Biomaterial microarrays for exploring polymer-stem cell interactions. (A) Monomers were mixed at a 70:30 ratio pairwise in all possible combinations. Monomer combinations were printed with a radical initiator onto a layer of poly(hydroxyethyl methacrylate) (pHEMA), on top of an epoxide-coated slide. (B) Day 6 embryoid bodies were dissociated and seeded onto polymer arrays in the presence of retinoic acid, the absence of retinoic acid and with a 24-h pulse of retinoic acid for 1 or 6 d. Cells were then stained for cytokeratin 7 (green), vimentin (red) and DNA (blue). (Figure and legend reproduced from Ref. 41 with permission.)
Fig. 6. Microwell platform for controlling the size of embryoid body (EB) formation. (A) Schematic of the process for generating microwell patterning Matrigel (MG) on a cell culture substrate, and subsequently seeding cells onto MG-patterned substrate. (B) To generate size-controlled micropatterned (MP) EBs, hESCs are dissociated to single cells and plated at high density onto patterned Matrigel islands and cultured to confluence. Intact colonies are then detached using a cell scraper and transferred to suspension in differentiation medium. Quantitative demonstration of EB size control in EBs generated from size-controlled human embryonic stem cell colonies (C) by EB diameter and (D) by comparing cell number in colonies to cell number in generated EBs. (E) The effect of micropatterned human embryonic stem cell (MP-hESC) colony size and cell composition on mesoderm and cardiac induction in MP-embryoid bodies (EBs). Gene expression levels measured for mesoderm markers Brachyury (Bry) and Mixl1, and cardiac marker α-Actin in day-8 (d8) EBs plotted with respect to the Gata6/Pax6 gene expression ratio in the corresponding MP-hESC starting population, and as a function of MP-hESC colony size demonstrate that mesoderm and cardiac induction of hESCs was significantly higher at larger EB sizes. (Figure and legend reproduced from Ref. 47 with permission.)
Directed differentiation of ESCs through formation of embryoid bodies (EBs) has been enhanced by using microwell array techniques. EBs are typically formed using the hanging drop method or in suspension culture. The resulting EBs are heterogeneous in shape and size. As a result, cell populations obtained from EBs formed by these methods can vary considerably. To provide more uniform microenvironments to EBs and thus more uniformly direct EB differentiation, microwell approaches were implemented (Figs. 6A and 6B). For example, poly(ethylene glycol) (PEG) microwells were used to create EBs of homogenous size and shape (Figs. 6C and 6D). More recently, such approaches were used to control hESC differentiation trajectories (Fig. 6E). Specifically, it was observed that mesoderm and cardiac induction of hESCs was significantly higher at larger EB sizes.

Microwell arrays were utilized for the parallel manipulation and quantitative analysis of stem cells at the single cell level. For example, combined biomimetic hydrogel matrix technology with microengineering was used to fabricate a microwell array which can be used to control NSC fate and neurosphere formation. Using this technology, the authors enhanced the viability and control the size of neurospheres formed from a single founding cell.

Microwell approaches provide precise control over microenvironmental parameters such as shape and size. However, in contrast to most multi-well formats, all wells in the microwell approach share the same culture media. Thus, it is difficult to vary other parameters of the microenvironment in a high-throughput manner.

3.2.4 Microfluidic array approaches to study stem cell biology

Microfluidic devices are powerful tools that have the ability to control the soluble and mechanical properties of the cell culture environment. Microfluidic devices are fabricated by the casting poly-dimethylsiloxane (PDMS) over a prefabricated-mold (Fig. 7A). The advantages of microfluidics include (1) decreased reaction rates and analysis times, (2) reduced consumption of reagents, (3) reduced production of harmful by-products, and (4) ability to run multiple experiments on a single chip.
Recently, microfluidic approaches were used for the analysis of signals that affect stem cell fate. A microfluidic device was developed for analyzing 16 unique mESC cultures in parallel.\textsuperscript{52} Using this platform, the authors identified an optimal flow rate that enhanced mESC colony formation, proliferation, and maintenance of pluripotency (Figs. 7B and 7C). Along similar lines, a micro-bioreactor array was fabricated using soft lithography that contains 12 independent micro-bioreactors.\textsuperscript{53} Each micro-bioreactor was perfused with independent culture media containing different biological molecules. Using this platform, a correlation between varying flow patterns and hESC differentiation into vascular lineages was established. Recently, an integrated microfluidic platform was designed that allows the screening of individual hESC colonies in real time using six individual cell culture chambers.\textsuperscript{54} Such approaches provided...
important information about the degree of hESC colony heterogeneity. Although these microfluidic platforms enable the multiplexing of experiments, they have the disadvantages of: (1) relative low throughput, (2) difficulty of fabrication, (3) inability to perform on chip immuno-cytchemistry, and (4) lack of compatibility with conventional, high-magnification light and fluorescent microscopy.

4. High-Throughput Intrinsic Systems for Stem Cell Investigations

Each of the microenvironment factors acts individually and in combination to perturb intrinsic cellular signaling networks which in turn influence stem cell functions and ultimately stem cell fate. As a result, a variety of HTS systems have been developed to manipulate the intrinsic signaling networks of stem cells. Such systems generally utilize large libraries comprised of small molecules, RNAi molecules or expression systems carrying shRNAs or cDNAs.

4.1 High-throughput RNA interference studies to investigate gene functions in stem cells

Elucidation of gene function is a critical aspect of advancing stem cell research. Functional genomics typically involve gain- and/or loss-of-function studies, which reveal the molecular mechanisms of a cellular phenotype, but these are difficult to implement at the genome-wide scale in cultured stem cells. Thus, most gene-silencing studies are restricted to knockout strains of model organisms such as yeast, flies, and mice.

Recent advances in RNA interference (RNAi) have aided the field of functional genomics by allowing for loss-of-function studies in mammalian cells without the need for germline inactivation of the gene being studied. RNAi occurs through the effect of the ribonuclease (RNase) enzyme Dicer on double stranded RNA. Dicer cleaves the dsRNA into double-stranded small interfering RNAs (siRNAs) which can act either through the RNA-induced silencing complex (RISC) to degrade complementary mRNA sequences or through the RNA-induced transcriptional silencing (RITS) complex to repress transcription and modify DNA and histone methylation.
RNAi screens have been used to study the effect of genetic control elements on stem cell behavior. For example, a subtractive RNAi library approach was used to identify multiple genes involved in the regulation of expression of Oct4 and of self-renewal. However, large-scale cell-based RNAi screens have been hampered by the demands and inefficiency of traditional HTS. Recently, RNAi cell microarrays were used for effective gene knockdown in hMSCs (Fig. 8). These technology platforms could offer an efficient approach for carrying out high-throughput loss-of-function studies. Arrays can be fabricated by spotting either lentiviruses that express short hairpin RNA (shRNA) to silence gene expression through RNAi or peptide transduction domain — double stranded RNA binding domains (PTD-dRBDs) that carry siRNA across the cell membrane and knockdown gene expression. Such RNAi arrays will aid in the rapid functional annotation of stem cell genomes and in the identification of genes involved in stem cell self-renewal and differentiation.

4.2 High-throughput generation of genetically modified stem cell lines

The ability to obtain information about gene function has been greatly aided by the generation of genetically modified stem cell lines. Several

![Fig. 8. siRNA microarray for high-throughput screening of gene function. (A) Cotransfection of an EGFP vector and anti-EGFP siRNA into hMSCs and co-transfection of an EGFP vector and scramble siRNA into hMSCs. (B) Quantification of the observed effects demonstrates the efficiency of the spotted siRNA gene knockdown. (Figure and legend reproduced from Ref. 59 with permission.)](image-url)
techniques have been implemented to create genetically modified stem cells in a high-throughput manner. For example, gene-trap mutagenesis, a technique that randomly generates loss-of-function mutations, was used to create more than 8000 mutagenized ES cell lines. More recently, clonal microarrays were used to create genetically modified stem cell lines (Fig. 9). Clonal microarrays are produced by seeding stem cells on microfabricated surfaces generated using soft lithographic techniques. Stem cells grown on these arrays can be infected with DNA constructs and then isolated after assaying in parallel for various parameters, such as proliferation, signal transduction, and differentiation.

5. Conclusions and Future Trends

The technologies presented here are capable of screening and perturbing several components of the stem cell microenvironment and can greatly advance the engineering of defined cell types from stem cells. However, additional factors, including oxygen and salt concentrations, mechanical
forces, matrix stiffness, and dimensionality, also play critical roles in the microenvironment and determination of cell fate. *In vivo*, the complex network of signaling and matrix molecules is subject to mechanical forces (such as pressure, fluid shear stress, and stretch), which play important roles in specifying embryonic polarity and tissue development. Recent studies have shown that application of shear stress to mESCs induces cell proliferation and endothelial cell (EC) lineage differentiation with the expression of marker genes indicative of ECs and the enhancement of endothelial functions. Application of cyclic stretch may induce differentiation toward smooth muscle lineage, and compression induces chondrogenesis. Substrate compliance is also known to influence cell fate decisions. For example, human mesenchymal stem cells (MSCs) showed higher rates of growth on stiffer substrates. Recently, it was demonstrated that lineage-specific differentiation of MSCs is induced by matrix stiffness that matches the respective tissue — soft matrices are neurogenic, rigid matrices are osteogenic, and intermediate matrices are myogenic. Finally, *in vivo*, cells often reside in 3D as opposed to 2D microenvironments. In fact, three-dimensionality has been shown to play a critical role in the microenvironment and can affect cell function. In the future, these high-throughput tools can be enhanced to screen such additional components, thereby creating more *in-vivo*-like screening conditions.

It has recently been demonstrated that stable genomic integration and high expression of four factors, Oct4/Sox2/Klf4/c-Myc or Oct4/Sox2/Nanog/LIN28, can reprogram fibroblast cells into induced pluripotent stem cells (iPSCs). This ability to generate patient-specific pluripotent stem cells will have a major impact in regenerative medicine. However, the use of iPSCs in cell-based therapies is limited because of the presence of virally transduced transcription factors and oncogenes. Furthermore, current protocols to establish iPSCs are extremely inefficient (<0.1%). The technology platforms presented here could be used to identify microenvironment components, such as ECMPs, growth factors, and small molecules, not only for the purpose of enhancing reprogramming efficiency, but also identifying factors that could replace the need for virally transduced transcription factors and oncogenes.

The HTS technologies described here could find usage in the pharmaceutical industry by aiding in crucial steps of drug development: toxicity
screening, target identification, and lead assessment. The current method of target identification involves the use of 96- or 384-well microtiter plates with monolayer cell cultures. However, the multi-well plate format suffers from several limitations, most notably the cost of the relatively large amounts of reagents and cells needed. The HTS technologies described here, by increasing the parallelism and efficiency of screening compound libraries, offer an attractive solution. Additionally, these technologies could be used to identify potentially toxic compounds earlier in the drug development process. For example, an array-based high-throughput system was recently developed that can be used to mimic the effects of human liver metabolism and simultaneously evaluate the cytotoxicity of small molecules and their metabolites. Along the same line, the use of high-throughput technologies in conjunction with stem cells and their differentiated progenitors could provide more realistic in vitro models for predicting the effectiveness and toxicity of drug candidates and chemicals in humans. Most cell lines currently used in drug screening paradigms are virally transformed cells of tumor origin that are not representative of the disease to be investigated. The derivation of hPSCs has made it possible, for the first time, to study various aspects of human development and disease using cells representative of these conditions.

In summary, hPSCs represent an infinite supply of cellular “raw-material” that can be used to generate more realistic disease models for drug discovery. The ability to manipulate hPSCs and adult stem cells using high-throughput technologies will enable the production of large amounts of specialized cells needed for applications in regenerative medicine and drug discovery.

References


1. Introduction

There is a severe shortage of tissues and organs for use in repair of diseased or otherwise defective tissues. Tissue engineering has emerged as a field to fabricate tissues in sufficient quantities to repair damaged tissues.\(^1\) While tissues such as skin\(^2\) or bone\(^3\) can repair a small injury in a reasonable amount of time, tissues such as cartilage\(^4\) and myocardium\(^5\) cannot regenerate, and will continue to degenerate and decrease in function without intervention.

Early “top-down” approaches comprised a majority of tissue engineering approaches, whereby biodegradable polymer scaffolds, such as poly (glycolic acid) (PGA),\(^6\) were seeded with the different cell types. In this approach, it was expected that the cells would proliferate and migrate throughout the scaffold and secrete the appropriate extracellular matrix (ECM) often aided by growth factors,\(^7\) perfusion,\(^6\) and mechanical\(^8\) or other stimulation.\(^9\) However, despite advances in scaffold technology, such as surface patterning\(^10\) or decellularization techniques for native
ECM structures, it is still difficult to recreate the intricate tissue microstructure necessary to create functional tissues.

The “bottom-up” approach aims to recreate the tissue microarchitecture by designing individual building blocks with specific microstructural features and assembling these blocks into larger engineered tissues. There are a number of techniques to fabricate these building blocks, such as fabrication of cell-laden microgels, self-assembled aggregation, creation of cell sheets or tissue printing technologies. After creating the building blocks, larger engineered tissues can be fabricated from these modules by a variety of approaches such as stacking, random packing or self assembly. The bottom-up approach attempts to mimic nature as many tissues are largely composed of repeated units with similar functions and architectures, including the lobule of the liver. Recapitulation of the native microarchitecture of naturally repeated units of tissues could lead to improved function at the microscale, creating biomimetic engineered tissues with improved functional properties.

One primary objective of bottom-up approaches is control of the cellular microarchitecture to better direct cellular function and ultimately tissue morphogenesis. In this respect, many microscale technologies and techniques used to create cell-laden building blocks can also be used as investigative models for determining and controlling cell behavior. These techniques have been employed recently for driving stem cell differentiation and function down specific lineages, which could be beneficial for use in a variety of tissue engineering and regenerative medicine applications. As many tissues are comprised of cells that typically do not undergo extensive self-replication, such as cardiomyocytes in cardiac tissue, the ability to control and dictate stem cell behavior is crucial to the future success of engineered tissues. In addition, recent research has confirmed the existence of stem cells residing in niches that are unique to the tissues and organs, which contain highly ordered microarchitectures and cellular compartmentalization and arrangement. Microscale technologies have the ability to recreate many of these complex features, giving great promise to creating in vitro microenvironments with the ability to quickly and effectively direct both adult and embryonic stem cell (ESC) behavior bringing us closer to the ultimate goal of clinical regenerative medicine applications.
The following chapter will highlight the current techniques for using microscale technologies both for creating engineered tissues using the bottom-up approach and for investigating and directing stem cell behavior. These two applications of microscale engineering are crucial to the future of regenerative medicine. In addition, as one field progresses and experiences new breakthroughs, similar analogous techniques can drive research in the other field. The future clinical success of regenerative medicine is highly dependent on successes in both tissue engineering and stem cell differentiation, suggesting that the more these fields can interact and use similar techniques the faster development will occur hopefully shortening the time needed to bring these techniques to the clinic.

2. Control of Cellular and Tissue Microarchitecture

The ability to control the cellular and tissue microarchitecture is key to both stem cell differentiation and bottom-up tissue engineering techniques. Through restriction of the cellular geometry, controlled environments can be created to investigate cell behavior.26 These geometrical restrictions can be performed by a number of methods, such as cell seeding in microscale channels27 or microwells28 or creating cell-laden hydrogels using micromolding.12

2.1 Hydrogels

Engineering an environment in which cell proliferation, differentiation and function can be tightly controlled is of great importance to regenerative medicine.29 As the cellular microenvironment has a profound effect on stem cell physiology, including proliferation and differentiation, the ability to precisely control the cellular microenvironment could allow for better control over directing stem cell behavior.30 Similarly, the cellular microenvironment, such as cell-cell and cell-ECM and cell-soluble factor interactions, have a substantial effect on the function and tissue morphogenesis of engineered tissues.31 Due to their characteristics, including biocompatibility, flexible methods of synthesis and a wide variety of physical characteristics, hydrogels have long been employed as scaffolding materials for tissue engineering.32
Hydrogels are composed of networks of hydrophilic polymer chains, which are the product of chemical or thermal interactions between pre-polymer chains. These chemical bonds could be permanent (such as covalent and ionic bonds) or temporary bonds (such as hydrogen bonds). Such structures make it feasible to control the crosslinking and network formation through manipulation of the reaction process, ultimately leading to better control over the microenvironment of the hydrogel. Adjusting the temperature of the hydrogel or manipulating the reaction energy by tuning the ultraviolet light (UV) exposure time and power are examples of such manipulations. In terms of ionic charges, based on the groups incorporated into the hydrogel backbone, hydrogels could be neutral, cationic, anionic or ampholytic. However, it is observed that charged hydrogels tend to have a greater potential for cell attachment as compared to neutrally-charged hydrogels. As a result, charged hydrogels are potentially better candidates for engineering tissue scaffolds. Many of the currently available hydrogels are synthesized from monomers which are either ionized or ionizable, improving the possibility of cell attachment and proliferation within cell-laden hydrogel scaffolds. Furthermore, incorporation of various cell-binding peptide domains, such as arginine-glycine-aspartic acid (RGD), into the hydrogel can dramatically increase cell binding and spreading within the gel.

The physical properties of hydrogels are another key factor making hydrogels attractive for tissue engineering applications. To optimize cell viability, having a microenvironment in which cells can easily exchange nutrients and waste products, oxygen and other soluble factors is of great importance. Diffusion is a key factor in solute transport in hydrogels, however in hydrogels with microscale pore structures or forced flow conditions, convection can be a critical factor as well. Experimental results have demonstrated that the hydrogels pH, temperature, mesh size and environmental conditions could significantly affect diffusion in ionic hydrogels. In addition, as will be detailed more thoroughly later in the text, hydrogels have been demonstrated to be highly amenable to a number of techniques to create structures with controllable features on the microscale. Microfabrication technologies have been applied to hydrogels to mimic the complex spatiotemporal in vivo ECM environment leading
to engineered tissues with biomimetic properties and microarchitectures. The ability to exert tighter control over the cellular microenvironment through microfabrication of hydrogels could greatly enhance the ability to control cell and tissue behavior ultimately leading to functional tissue structures.

2.2 Cell seeding in microwells

Control of cell aggregation through seeding in fixed geometrical spaces is an effective means of restricting aggregate size and dimensions to direct tissue morphogenesis. Seeding cells in non-adherent microwells formed spheroid structures, while larger organoid structures were created by culturing cells in linear channels leading to improved cell alignment and resulting tissue function. A major advantage of these techniques, for compatible cell types, is the ability to allow the cells to remodel and recreate the microarchitecture using only natural materials while allowing the cells to dictate the pace and organization of the tissue morphogenesis.

Taking this one step further, researchers have used micromolds to generate shape-controlled cellular aggregates, in geometries such as spheroids, individual and connected tori or honeycomb structures. Cells were then seeded onto the molded hydrogels, leading to self assembled cellular organization within the shape restricted templates.

2.3 Cell-laden hydrogels

To create microtissues researchers combine cells with hydrogels to create tissue-like structures of specific mechanical properties and geometries. Some common techniques mix cells within polymer hydrogels, such as poly (ethylene glycol) (PEG) or other photopolymerizable materials, self-assembling peptide gels or temperature sensitive hydrogels. In this approach, cells are combined with the hydrogel precursors, pipetted into micromolds and polymerized using incubation or UV light. An alternative approach creates cell-laden hydrogels by directly passing UV light through a patterned mask containing specific shapes, which, when optimized, only polymerizes the hydrogels where the UV light is able to penetrate the mask.
One example of using cell-laden hydrogels for tissue engineering created rings of primary cardiac cells within molded collagen or Matrigel. These cell-laden rings were arranged in contact with other rings and cultured under cyclic mechanical stretch forming a composite tissue, with improved contractile function and cell alignment. \textit{In vivo} implantation of these tissues in a rat myocardial infarct model displayed significant improvements in cardiac function demonstrating the ability to create functional tissues using assembled macroscale cell-laden hydrogels.\textsuperscript{5}

\subsection*{2.4 Microarray systems}

Microscale technologies have emerged as a powerful tool for high-throughput cellular and biological studies as well as a means for manipulating biological systems and miniaturizing experiments.\textsuperscript{44} A microarray is a fabricated device composed of a specified number of microwells of defined shape and size which can be used to culture cells and/or to deposit combinations of different materials such as collagen, laminin, and fibronectin to enable the study of cell behavior in a high-throughput manner. These miniaturized arrays can be used to segregate cells for single cell analysis and multiple cells studies, such as forming embryoid bodies.\textsuperscript{45} One major advantage of microarray systems is that the throughput is significantly higher than is possible using traditional cell culture techniques as these arrays can contain hundreds or thousands of microwells. In addition, due to the microscale size, expensive reagents and soluble factors, such as growth factors, cytokines or drugs, are conserved making large-scale combinatorial experiments feasible. Using time-lapse microscopy and immunostaining, the fate of several hundred single stem cells can be tracked simultaneously making this a powerful tool for \textit{in vitro} determination of the impact of exogenous factors on cell and tissue behavior.

\section*{3. Microscale Technologies to Investigate and Control Stem Cell Behavior}

The derivation of ESCs from both mouse and human\textsuperscript{46} has opened up new possibilities for cell-based therapies. However, the use of human embryos to generate ESC lines is controversial and therefore recognized as
ethically problematic.\textsuperscript{47} A breakthrough to this problem is in the form of reprogrammed somatic cells.\textsuperscript{48} These induced pluripotent stem (iPS) cells have been shown to be functionally and molecularly similar to ESCs\textsuperscript{49} and offer new opportunities in regenerative cell therapy applications.\textsuperscript{50} The ability to reliably direct stem cell differentiation to ectodermal, mesodermal, and endodermal lineages is a potentially powerful method for generating functional tissues, as tissues emerge from well-organized sequences of cell renewal, differentiation, and assembly under normal physiological conditions.\textsuperscript{51}

Stem cells are sensitive to a variety of microenvironmental stimuli that regulate both self-renewal and differentiation.\textsuperscript{52} Thus, through microscale engineering, cell differentiation can be guided by controlling the interplay between regulatory factors down to the individual cellular level.

3.1 \textit{Microarray analysis of ESC differentiation}

ESC differentiation can be directed through different combinations of factors that influence the microenvironment. Spatiotemporal microenvironment signals may be influenced by the size of embryoid bodies (EBs), co-culture of different cell lineages, small molecules present in the microenvironment and ECM materials in combination with other unknown parameters. To study the effect of all stem cell differentiation factors is an enormous combinatorial problem that would be extremely difficult to analyze without the facilitation of high-throughput analysis, screening, and imaging. This goal can be achieved much more readily through the use of microarray systems for analysis and screening of multiple factors individually and in combination. Because of the significant number of possible combinations, high-throughput approaches can be used to rapidly test and discover important combinations of parameters in ESC differentiation. Parameters such as ECM molecules, soluble factors (e.g. cytokines, growth factors), and biomaterial interactions can influence various biological pathways that influence ESC differentiation. To improve our understanding of ESC differentiation, studying the combined influence of parameters that may regulate stem cell expansion and specialization in an efficient and
cost effective manner using high-throughput analysis, screening, and imaging may prove beneficial.

In addition to growth factors and cell-secreted factors, synthetic small molecules can direct ESC differentiation. Small permeable, naturally occurring molecules such as vitamin C, sodium pyruvate, dexamethasone, thyroid hormones, and retinoic acid have been used to regulate stem cell fate. Similarly, new synthetic, heterocyclic small molecules that can alter stem cell fate have recently been studied to control stem cell differentiation. Ding et al. reported ESC differentiation into various cell types employing small molecules. One manner by which synthetic molecules can be used to selectively control and regulate stem cell differentiation and proliferation is through adjusting the activities of proteins. Such processes have been successfully employed to cause controllable neurogenetic and cardiomyogenetic induction in murine ESCs, osteogenesis induction in mesenchymal stem cells (MSC), and skeletal muscle cell differentiation. Further investigation into small molecule discovery could play a substantial role in furthering the ability to reliably differentiate stem cells down specific pathways.

The interactions between ESCs and the surrounding matrix environment can profoundly influence stem cell behavior. To assess this parameter Anderson et al. used a biomaterial library comprised of 576 different acrylate-based polymers to analyze stem cell behavior. Combinations of the different polymers were mixed in 384-well plates and were robotically printed on coated glass slides. After printing, the slides were exposed to long wave UV to initiate polymerization, dried, sterilized, and washed with PBS and cell culture media. Embryonic stem cells were then seeded onto the slides and the influence of the materials on their differentiation was analyzed. Based on these experiments, it was concluded that ESC differentiation may be induced toward epithelial cell lineage through interactions with specific combinations of materials. This study demonstrated the potential for creating a library of materials to study ESC-material interactions which could be used to better design scaffolds or culture conditions for controlling the behavior of ESCs, or other stem or differentiated cell types.
3.2 Microwell fabrication

As briefly described above, microengineered wells can be a useful tool to control the geometry and homogeneity of cell aggregates. To fabricate microwell arrays, UV-photocrosslinkable PEG prepolymer containing photoinitiator can be placed on a glass slide that has been treated to be adhesive to the hydrogel. The precursor solution was crosslinked between the surface-treated glass slide and a coverslip with a photomask on top to control the spatial geometry of the crosslinking (Fig. 1). Microscopy cover slips were used as spacers between the glass support and the coverglass to define

Fig. 1. (Left) PEG microwell fabrication through UV crosslinking. (Right) Localizing cells within arrays of microwells using a wiping technique. This method produces cell seeding densities that vary consistently with microwell geometry and cell concentration. (Copyright (2009) Wiley. Used with permission from Ref.63.)
the depth of the microwells. The PEG precursor solution was then irradiated through a bright field photomask with UV light of 350–500 nm to generate the microwells. The PEG precursor solution only underwent polymerization in the areas where UV light was able to pass through the photomask, while all other areas remained in the liquid prepolymer state. Following polymerization of the polymers, the coverglass was carefully removed and the uncrosslinked PEG macromers were removed with deionized water.

3.3 Microfluidic systems

The field of microfluidics could be useful for controlling the interaction of stem cells with their surrounding soluble environment as it allows manipulation of microliter volumes of fluids within microchannels. Microfluidic systems have been used in a number of different applications for controlling the cellular microenvironment such as micropatterning of cells, subcellular localization of media components, high-throughput drug screening, introduction of a large range of laminar-flow rates, and creation of soluble factor gradients. A unique aspect of microfluidic systems is the ability to control mixing and shear stress in which the microfluidic devices can produce a logarithmic scale of flow rates and a logarithmic concentration gradient. This ability has been utilized to study ESCs where high flow rates resulted in increased proliferation with minimized usage of media as compared to traditional macroscale experiments. This important advantage may be applied for the screening of media components, conditioned media, or different chemical formulation with minimized consumption and optimized cell behavior. The use of microfluidic systems in controlled flow rates or concentration gradients have been shown to be important for regulating cell proliferation and differentiation.

3.4 Controlled microbioreactors

One of the main challenges in regenerative medicine is obtaining ample amount of specific cell types for capable transplantation. However, these efforts have been hindered by limited proliferative capacity of the desired cells. Incorporation of bioreactor systems with ESCs is an active area of
investigation since ESCs can be differentiated into cell lineages of all three primary germ layers. ESCs can greatly benefit from bioreactors as biological processes can be carried out under tightly controlled (oxygen, nutrients, or other molecular and physical regulatory factors) environmental conditions to account for controlled nutrient transfer. These conditions will help minimize the batch to batch variability, making the process sufficiently reproducible in regenerative medicine. Bioreactor systems may better facilitate the transition from laboratory to clinical scale production due to high level of control, while providing a means to quantitatively study cell behavior in response to various stimuli. Some of the essential requirements to be considered during design are: (1) rapid and controllable expansion of cells; (2) enhanced cell seeding of 3D scaffolds (at a desired cell density, high yield, high kinetic rate, and spatial uniformity); (3) efficient local exchange of oxygen, nutrients, and metabolites; and (4) implementation of physiological stimuli.

4. Assembly Techniques for Creating Engineered Tissues from Microscale Building Blocks

Just as ESC behavior can be directed and manipulated through microscale technologies, engineered microtissues can be engineered by microgels with specific microarchitectural features. The field of bottom-up tissue engineering aims to create macroscale engineered tissues with tightly controlled microarchitectural structures to better recapitulate the native structure and function of the tissues we are aiming to repair or replace. The major challenge of tissue engineering using bottom-up techniques is assembling macroscale engineered tissues from microscale building blocks. For optimal function in vivo, engineered tissues must recreate the native microarchitecture, while also possessing robust mechanical properties for appropriate interactions with the surrounding tissues and to withstand the mechanical environment. Some specific challenges to overcome for this field are: determination and recapitulation of native mechanical properties, building block integration to form robust tissues, creation of integrated microvasculature, scale-up technologies to move from the laboratory to the clinic at the appropriate length scale and
successful demonstration of *in vivo* functional improvement in diseased tissues.

### 4.1 Layer by layer production using cell-laden hydrogels

Layer by layer assembly of tissue sheets can be used to generate larger 3D structures that mimic tissues. In one approach to create engineered tissues using this layer by layer approach, arrays of cells and ECM/polymer were photocrosslinked in an additive manner. Through additive photopolymerization, multilayer engineered tissues were fabricated for hepatic tissue engineering using cell-laden hydrogels. Hepatocytes from adult Lewis rats were mixed with PEG prepolymer that had been functionalized with cell-adhesive RGD motifs. The cell-PEG mixture was pipetted into a custom designed sterile chamber and polymerized through the application of UV light exposed through one of three photomasks, the first of which was in the shape of three pointed stars. Next, the spacer height was increased to create a second subsequent layer which extended the full spacer height partially resting on top of the initial layer (Fig. 2). Finally, the spacer height was increased a final time, and a honeycomb enclosure was created around each two-layer unit, creating engineered microtissues with microarchitecture and function similar to native hepatic tissues. Using this technique Bhatia and colleagues were able to demonstrate many benchmarks of functional hepatic tissue, such as urea production, while also greatly improving hepatocyte adhesion both by inclusion of RGD molecules as well as through recapitulation of the native microarchitecture of the liver lobule. While there are many clear advantages of this technique, such as recreation of the native microarchitecture combined with positive functional properties similar to the native tissues, one potential problem with this technique is the inability to tightly control the layer thickness. There is no simple method for restricting subsequent cell-PEG mixtures from filling the chamber, making it difficult to have multiple layers that only occupy one plane. For some tissues using different cell types on each layer, this technique would have difficulty recapitulating the native cellular arrangement. Clearly the positive aspects outweigh the negative, however in an effort to create more complex patterns and mimic more
complex tissues, addressing these shortcomings could make this technique applicable to more tissues and applications.

4.2 Packing of co-cultured building blocks for capillary filtration

A major challenge in creating engineered tissues that can function and integrate in vivo, is the ability to create tissues containing a functional microvasculature to integrate with and ensure perfusion through and around the surrounding tissues. Recently an approach has been demonstrated that aims to create a perfusable tissue without creating microvasculature.
To create perfusable tissues analogous to capillary filtration, a random hydrogel packing technique was developed to create tissues with tortuous, perfusable networks of capillaries. Cylindrical tissue units were created through packing of cell-laden collagen hydrogel building blocks within perfused silicon tubing. To create the building blocks, collagen was mixed either with HepG2 cells, a model hepatic cell line, or without. Following gelation in cylindrical tubing, cylindrical cell-laden building blocks were created by chopping the long cylindrical tissue into pieces. The cell-laden gels were collected in media, then were combined with HUVEC cells to form a confluent endothelial layer around the perimeter of the hydrogel blocks. The HepG2-HUVEC modules were perfused into tubing containing a porous plug to both trap the gels and also to allow the modules to aggregate and compact together. Over time the co-cultured constructs compacted further and remodeled together to form a porous, perfusable tissue. The engineered filtration devices were perfused with blood to demonstrate both high viability of the encapsulated HepG2 and the surface coated HUVEC cells, and the ability to perfuse without clotting, for use as clinical blood filtration devices (Fig. 3). While, this relatively simple technique for creating perfusable tissues could potentially be effective in replacing tissues which primary function as filtration systems, such as the kidney or liver, other tissues would potentially not be possible due to the lack of a self-contained enclosure as well as poor mechanical

**Fig. 3.** Packing of cell-containing building blocks in a perfusable reactor. HepG2 liver cells were encapsulated in collagen, cast into modular units then coated with a confluent layer of HUVECs and packed inside a perfused tube to generate a perfusable filtration unit. (Reprinted with permission from the *Proceedings of the National Academy of Sciences USA.*20)
properties. In addition, the encapsulated cells would have to retain their viability and function while being surrounded by endothelial cells, potentially limiting the available cell types appropriate for encapsulation.

4.3 Directed assembly of cell-laden hydrogels

Recent work in our laboratory has used directed assembly to demonstrate the ability to create ordered tissue structures using cell-laden microgels as building blocks. This technique demonstrates one method by which higher order structures can be created when the building block materials are fragile or difficult to handle. In addition, this technique also demonstrates the potential for scale-up or automated techniques, as the engineered tissues can be created almost entirely without intervention.

This technique was made possible by harnessing the properties of surface tension with regard to hydrophilic hydrogels to assemble cell-laden building blocks into tissue-like structures (Fig. 4). For the first step, rectangular PEG hydrogels of varying aspect ratios were created containing encapsulated cells through direct UV photopolymerization using specially designed photomasks. These building blocks were then placed into mineral oil, which is hydrophobic, to cause the hydrophilic microgels to aggregate. These aggregates were then exposed to a brief, second UV exposure to crosslink the assembled microgels. The ultimate shape and overall dimensions of the tissues were demonstrated to be controllable to a certain extent based on the aspect ratio of the building blocks used, with the total number of microgels increasing with the aspect ratio. Even greater control of the process, and the potential for making more complex structures, was demonstrated through use of spatially controllable lock and key geometries, suggesting a potential way to further increase the size and intricacy of tissues created using this technique. While there are clear advantages to this technique, such as the ability to create tissues of specific sizes based on the geometries of the building block materials and without complicated manipulation or assembly techniques, there are also potential drawbacks to this technique. For instance, the ultimate size of the assembled tissues was on the order of millimeters, making a clear clinical application difficult to envision, unless these tissues were used as the basis for a secondary assembly process. In addition, a large fraction
Fig. 4. Directed assembly of shape controlled hydrogel building blocks using a two-phase reactor. Lock (A) and key (B) shaped hydrogel modules were created directly by photopolymerization using UV through a photomask, then allowed to aggregate and self-assemble in a hydrophobic media (mineral oil), into single (C, D), double (E, F) and triple (G, H) arrangements demonstrating the possible control of assembled co-cultured structures. Scale: 200 \(\mu\)m. After assembly a second UV polymerization solidified the structures. (Reprinted with permission from the Proceedings of the National Academy of Sciences USA.\textsuperscript{14})
of the microgels assembled randomly instead of in a controllable manner, suggesting that optimization must be performed to reduce the variability in the system. Current research into adapting this technique to be performed on a surface, rather than as a 3D immersion, are beginning to overcome many of these challenges and show great promise towards creating engineered tissues with controlled co-culture on a clinically relevant length scale.

5. Conclusions and Future Directions

Some major challenges affecting both the fields of microscale tissue engineering and stem cell differentiation in the future are improving the overall spatial resolution leading to smaller size structures with enhanced biomimetic functionalities. Control on an even smaller scale of structures and stem cell will allow the investigation of interactions on a broader spectrum than what is possible with current techniques. The major challenge for bottom-up tissue engineering approaches will be the improving the ability to create tissues with controllable microarchitectures, yet, on a clinically, and biologically, relevant length scale for \textit{in vivo} implantations. One of the greatest challenges of microscale stem cell technologies is the ability to direct proliferation and differentiation more reliably. The combination of these fields contains great potential for the advancement of the field of regenerative medicine. The improvements in the fields mentioned above will make the possibility of regenerating degenerated or defective tissues much closer to reality, providing hope for millions of current and future patients with no other alternatives for cure.

References


1. Introduction

Cell- and tissue-based therapies can be broadly defined as the treatment of human diseases using human or animal cells. Original examples of such therapies were introduced with the intent to restore the blood and immune system of patients. In its simplest version, this approach dates back to the first successful blood transfusion in 1818 by James Blundel. Later, E. Donnall Thomas performed the first syngeneic (1954) and then allogeneic (1969) bone marrow transplants, pioneering work for which he received the Nobel Prize in Physiology and Medicine in 1990.\textsuperscript{1,2} More recently, stem cells of a different origin have been successfully used for similar purposes.\textsuperscript{3} Varying the nature of the starting cells or tissue and the method or extent of any subsequent processing has generated a stunning range of applications for cell- and tissue-based therapies. Some of these applications currently include diabetes regulation, osteoarthritis treatment, immune system modulation, regeneration of neural cells through injection of stem cells into the spinal cord, and replacement of whole organs (cornea, skin, blood vessels) through tissue engineering.
Cell sources for cell- and tissue-based therapies include differentiated cells extracted from the tissues of living adult humans or cadavers, adult stem and progenitor cells, and embryonic and fetal stem cells. For most applications, the cells or tissues used require some level of \textit{ex vivo} processing. Some applications require only minimal manipulation for cell purification and storage. For others, a more extensive manufacturing process may include the proliferation, differentiation, selection, pharmacological treatment, or genetic modification of the cells. \textit{Ex vivo} modifications are made to expand a specific cell type, restore a specific cell function, or build a specific tissue structure from the cells (tissue-engineered products). Personalized cell- and tissue-based therapies add to this level of complexity by customizing the cell-based treatment to individual patient needs.

Regulations are in place to ensure the safety of the patients by controlling the quality of the cell- and tissue-based product. They aim to control all aspects of the manufacturing process from the selection of the cell and tissue source, to its extraction and subsequent processing, and ultimately its implantation or re-injection into the patient. The spectrum of possible cell- and tissue-based therapeutic applications is wide enough that regulations applying to the quality control of one application might not apply to another. \textit{This chapter will focus on the quality control of therapeutic products derived from autologous cells or tissues and requiring extensive \textit{ex vivo} manipulation}. Such products meet the FDA’s definition of autologous somatic cell therapy products.\cite{4}

\section{2. Regulations Pertaining to Quality Control of Cell- and Tissue-Based Products}

Regulations pertaining to quality control of autologous cell- and tissue-based product manufacturing are based on preexisting regulations designed for drugs and pharmaceutical products. These preexisting regulations were adjusted to meet the specific challenges associated with the manufacture of therapeutic products derived from cells or tissues manipulated \textit{ex vivo}. Not surprisingly, the regulatory burden varies with the perceived risk associated with each product.
In the current Good Tissue Practices (cGTP) Final Rule (21 CFR 1271), the last version of which was issued in 2005, the FDA categorized cell- and tissue-based products into three classes based on their relative safety risk. This risk assessment is based on the extent to which the required processing alters the initial biological characteristics of the cells or tissues used in the product. Products requiring only minimal manipulation and where the cells or tissues perform the function for which they were biologically intended are considered low risk and are not subject to 21 CFR 1271 rules.

In contrast, cell- and tissue-based products that either:

- require significant \textit{ex vivo} manipulation such as cell expansion through tissue culture,
- are combined with another article,
- are intended for a use that is different from their initial biological purpose,
- or have a systemic effect and are dependent upon the metabolic activity of live cells for their primary function,

are considered high-risk and are subject to more stringent regulations (see Table 1 for more details on three-tiered cGTP classification). Products derived from autologous cells or tissues and requiring extensive \textit{ex vivo} manipulations belong to this high-risk category. As such, these products are regulated as biological drugs under the Federal Food, Drug, and Cosmetic Act and Section 351 of the Public Health Services (PHS) Act (42 U.S.C. 262, “351 products”). They must be evaluated through a Biologic License Application (BLA) pathway before they reach the market, an Investigational New Drug (IND) application must be granted by the FDA before they are clinically tested, and their manufacturing process is subject to current Good Manufacturing Practices (cGMP) (21 CFR 210 and 211) and applicable parts of 21 CFR 1271 (subparts A, C and D). They are also subject to the various regulations listed in Tables 2 and 3.

Similar pathways and risk assessment-based classifications exist in Europe where autologous cells or tissues manipulated \textit{ex vivo} and intended for medical applications are labeled as Advanced Therapy Medicinal Products.
Table 1. Three Tiered Approach of cGTP Final Rule (21 CFR 1271).

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<th>Risk Assessment</th>
<th>Criteria Met by Products</th>
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<td>Low risk, not subject to 21 CFR 1271 rules</td>
<td>Requiring only minimal manipulation and are meant to replace a function for which the cells or tissues were biologically intended (homologous use)</td>
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<tr>
<td>Higher risk, subject to 21 CFR 1271 rules and Section 361 of PHS* Act</td>
<td>All of the following:</td>
</tr>
<tr>
<td></td>
<td>• Minimally manipulated</td>
</tr>
<tr>
<td></td>
<td>• Intended for homologous use only</td>
</tr>
<tr>
<td></td>
<td>• Manufacture does not involve combining cells or tissue with another article, except for water, crystalloids, sterilizing, preserving, or storage agents that do not raise new clinical safety concerns</td>
</tr>
<tr>
<td></td>
<td>• Either:</td>
</tr>
<tr>
<td></td>
<td>— Does not have a systemic effect and is not dependent upon the metabolic activity of live cells for their primary function; or,</td>
</tr>
<tr>
<td></td>
<td>— Has a systemic effect and is dependent upon the metabolic activity of live cells for its primary function, and is for:</td>
</tr>
<tr>
<td></td>
<td>a. Autologous use</td>
</tr>
<tr>
<td></td>
<td>b. Allogeneic use in a first- or second-degree blood relative, or</td>
</tr>
<tr>
<td></td>
<td>c. Reproductive use</td>
</tr>
<tr>
<td>High risk, subject to 21 CFR 1271 rules and Section 351 of PHS* Act</td>
<td>Any of the following:</td>
</tr>
<tr>
<td></td>
<td>• More than minimally manipulated: expanded, activated, genetically modified</td>
</tr>
<tr>
<td></td>
<td>• Combined with another article (except some preserving and storage reagents)</td>
</tr>
<tr>
<td></td>
<td>• Intended for a use that is different than its initial biological purpose</td>
</tr>
<tr>
<td></td>
<td>• Has a systemic effect and is dependent upon the metabolic activity of live cells for its primary function, and is not for:</td>
</tr>
<tr>
<td></td>
<td>a. Autologous use</td>
</tr>
<tr>
<td></td>
<td>b. Allogeneic use in a first- or second-degree blood relative, or</td>
</tr>
<tr>
<td></td>
<td>c. Reproductive use</td>
</tr>
</tbody>
</table>

*Public Health Services Act.
3. cGMP, cGTP and Quality System

The objective of cGMP and cGTP is to ensure patient safety by controlling the *identity, purity, stability, viability, and consistency* of both the intermediate and final products. In order to ensure compliance with cGMP and cGTP requirements, every establishment processing “351 products” must devise its own *Quality System*. This system is a comprehensive program developed by the investigator or manufacturer and is tailored to monitor specific cell- and tissue-based products. According to 21 CFR 1271 (1271.3 (hh)), this program should be designed, among other things, to *prevent, detect, and correct deficiencies* that might increase the risk of introducing, transmitting, and spreading communicable diseases. While

<table>
<thead>
<tr>
<th>Regulation</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 CFR 312</td>
<td>Investigational New Drug (IND) Application</td>
</tr>
<tr>
<td>21 CFR 210/211</td>
<td>Current Good Manufacturing Practices</td>
</tr>
<tr>
<td>21 CFR 50</td>
<td>Protection of Human Subjects</td>
</tr>
<tr>
<td>21 CFR 56</td>
<td>Institutional Review Boards</td>
</tr>
<tr>
<td>21 CFR 1271</td>
<td>Subparts A, C, and D</td>
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</tbody>
</table>

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<tr>
<th>Regulation</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 CFR 201</td>
<td>Labeling</td>
</tr>
<tr>
<td>21 CFR 202</td>
<td>Advertising</td>
</tr>
<tr>
<td>21 CFR 210/211</td>
<td>Current Good Manufacturing Practices</td>
</tr>
<tr>
<td>21 CFR 600</td>
<td>Biological Products; General (includes Reporting of Adverse Experiences and Biological Deviations)</td>
</tr>
<tr>
<td>21 CFR 610</td>
<td>General Biologics Standards</td>
</tr>
<tr>
<td>21 CFR 1271</td>
<td>Subparts A, B, C, and D</td>
</tr>
</tbody>
</table>
there are multiple organizational variations, the quality program typically includes:

- A set of *Standard Operating Procedures* (SOPs): written documents that describe all activities performed.
- A *Quality Control Program*: a program that monitors certain characteristics of the process and product.
- A *Quality Assurance Program*: a program that utilizes audits of records, revision of SOPs, personnel training, deviation monitoring and investigation, implementation of corrective and preventive actions (CAPA), and review of the final-product release process to ensure that all activities are performed according to SOPs and that all quality control results meet preset criteria.

Figure 1 succinctly illustrates how these three basic elements interact to control the manufacturing and clinical testing of cell- and tissue-based products.

4. **Core Requirements of a Quality Program**

4.1 **Facilities**

(§ 1271.190(a) and (b)): Any facility used in the manufacture of cell therapy products must be of suitable size, construction, and location to prevent contamination of the product with communicable disease agents and to ensure orderly processing without mix-up. The facility must be designed so that each manufacturing step has a dedicated area, whose cleanliness matches the safety risk associated with that step. A cleaning program, supported by appropriate environmental monitoring, must be developed, implemented, and carefully documented. The flow of supplies, personnel, products, and wastes in the facility must be conceived so as to minimize the risk of contamination or cross-contamination of any of the products manufactured in the facility.

4.2 **Environmental control and monitoring**

(§ 1271.195(a)): Where environmental conditions could reasonably be expected to cause contamination or cross-contamination of the
product or equipment, they must be adequately controlled. The following control activities or systems should be provided as needed: temperature and humidity controls, proper ventilation and air filtration, adequate cleaning and disinfection of rooms and equipment to ensure aseptic processing, and maintenance of equipment used to control conditions necessary for aseptic processing operations. Documents such as the FDA’s Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing-cGMP and the EU’s Annex 1 (from Guidelines to GMP for Medicinal Products for Human Veterinary Use) can be used to determine the appropriate level of control needed in each production area. This level will depend on

![Diagram of Quality System: QA + QC](image)

**Fig. 1.** This figure illustrates how the Quality System monitors the manufacturing and clinical testing of cell- and tissue-based therapy products. It also depicts how its three basic elements: Standard Operating Procedures (SOPs), Quality Assurance (QA), and Quality Control (QC) function together within the Quality System framework. Activities typically controlled by QA and QC are highlighted in red and blue, respectively.
factors such as the manufacturing steps involved, whether they are performed in an open or closed system, etc.

Monitoring of controlled environments must be performed regularly, but the appropriate frequency is defined by the manufacturer or investigator. Monitoring can include non-viable and viable particulate air monitoring, clean-are positive pressure levels, as well as surface and personnel monitoring. USP 〈1116〉¹⁶ and ISO 14644-2¹⁷ contain useful information to help determine the type, method, and frequency of environmental monitoring needed for any specific controlled environment. Records of environmental control and monitoring activities must be maintained. It is important that relevant procedures define alert and action levels for environmental monitoring results and specify relevant corrective actions when these levels are exceeded.

4.3 Equipment

(§ 1271.200(a)): Equipment design, location, and installation should facilitate all operations, including those pertaining to maintenance and cleaning. Cleaning, sanitizing, and maintenance of all equipment should be performed according to well-defined procedures and schedules. All equipment used for inspecting, measuring, or testing must be demonstrated to be capable of producing valid results and calibrated according to established procedures and schedules. This includes, for example, equipment used to monitor the temperature of a storage unit or equipment used to measure air particle counts inside a controlled environment. Calibration accuracy should be in accordance with accepted standards (e.g. National Institute of Standards and Technology). Although § 1271.200 does not specifically require equipment qualification and certification, doing so is strongly recommended, particularly as part of process validation.¹⁸–²⁰ Equipment in which aseptic operations are performed, e.g. laminar flow hoods and biological safety cabinets, should be certified according to a strict schedule. Records of maintenance, cleaning, sanitization, calibration, certification, and use for each piece of equipment used during the manufacture of any batch of product should be kept on file.
4.4 Supplies and reagents

(§1271.210 (a) and (b)): All materials used during manufacturing, whether or not they come in direct contact with the processed product, are considered supplies or reagents. Examples of supplies include sterile drapes, gloves, pipettes, cell culture flasks, and shipping containers. Examples of reagents include culture medium, saline, antibiotic solutions, cleaning agents, and chemicals used in processing.

A system must be implemented that will ensure that supplies and reagents are not used until they have been verified to meet predetermined specifications. These specifications are designed to diminish the risk of introducing, transmitting, or spreading communicable diseases, and to prevent toxicity. The establishment using the supply or reagent can either verify that it meets the requisite specifications through relevant testing or by obtaining proper documentation from the manufacturer (typically a Certificate of Analysis for reagents or a Certificate of Compliance for supplies). Each supply or reagent must go through an approval process during which accompanying documentation is checked to verify that it corresponds to the predetermined specifications. Prior to inspection, items should be stored in a quarantine location according to manufacturer instructions. Accepted items must be moved to areas dedicated to the storage of approved items (1271.26(a–d)). They should then be stored and used according to the manufacturer instructions. Reagents produced in house should also undergo a verification process to ensure they meet the predetermined specifications (e.g. solutions are sterile, have the proper concentrations, and fall within a specific pH range). The water used throughout the manufacturing process (to rinse equipment or parts or prepare reagents) must be of appropriate cleanliness and, if produced in the facility, properly monitored. To limit the possibility of endotoxin contamination, water used for preparing reagents is usually Water for Injection, USP grade. Processes used to produce these reagents should be validated.

Keeping information data sheets listing specifications for all supplies and reagents is a good way to ensure consistency during the approval process. These sheets should be updated when products change and an archive of previously used data sheets should be kept. Under §1271.210 (d), records must be maintained for receipt of each supply or reagent, including
the type, quantity, source, lot number, date of receipt, and expiration date. Records of the approval process for each supply or reagent, including test results or Certificates of Analysis from the vendor, should be kept on file. Records of all lots of supplies or reagents used in the manufacture of each batch of product should also be kept.

4.5 Manufacturing process validation

The ability of an established manufacturing process to effectively and reproducibly provide intermediate and final products of predetermined specifications must be documented through adequate validations. There are three possible validation types: prospective, concurrent, or retrospective. Prospective validation is the preferred approach and should be completed prior to commercial distribution of the final product. The number of process batches to be included in a validation is a function of the complexity of the manufacturing process. For prospective and concurrent validation, three consecutive, successful production batches should typically be used. More batches may be necessary to prove the consistency of a manufacturing process if it is particularly complex or requires prolonged completion times. For retrospective validations, data from ten to 30 consecutive batches should generally be reviewed.

Critical parameters should be controlled and monitored during process validation studies. Such studies should include all critical process steps and be performed using qualified equipment and facilities according to a written validation protocol. The quality assurance team should regularly review validated processes. If the reviews confirm that the process consistently produces products meeting specifications, no revalidation is normally needed.

Additionally, the aseptic status of a manufacturing process should be validated through a medium fill simulation. This is done by simulating each of the critical manufacturing steps using a growth medium (broth) that promotes the growth of bacteria and fungi instead of any liquid reagent regularly used. The broth is subsequently incubated and observed to detect any microbial growth. Whenever feasible, simulations should be performed at the end of a production shift to capture the worst-case scenario conditions in terms of microbial contamination risk. For the
initial (start-up) validation, three medium fill simulations should typically be performed per manufacturing step. One simulation per manufacturing step can be performed during each subsequent validation. Subsequent validations should be performed once every six months.

5. Tailoring Quality Control to the Manufacturing Process

5.1 Quality control: Safety, consistency, and traceability

A proper quality program should address critical safety issues specific to the various steps of the manufacturing process. This is easiest to achieve when the quality system framework is developed in parallel with the manufacturing process during the product development (a concept coined as “Quality by Design”). This is realized by thoroughly assessing the safety risk associated with each manufacturing step and by implementing specific risk control measures to reduce the identified risk to acceptable levels. The adequacy of the risk control measures should be regularly reviewed as part of risk control management. Importantly, the level of risk associated with the manufacturing process and the resulting final product must be communicated to all interested parties (including regulatory authorities and patients, when relevant) throughout the product life cycle.

To ensure consistency, proper ways to execute and control each manufacturing step must be described in written documents, called Standard Operating Procedures (SOPs). It is also critical that personnel be regularly trained to follow SOPs. Furthermore, every step of the manufacturing process, as well as all results from quality control tests, should be documented in manufacturing Batch Records that undergo regular scrutiny by the quality assurance staff (21 CFR 211.188) and must be thoroughly reviewed as part of the process leading to the final product release (21 CFR 211.192). According to 21 CFR 211.188, batch records should include full traceability of patient material through all steps of the manufacturing process, including initial biopsy, final-product shipping, and eventual disposal. They should also contain the identification of all equipment, reagents, and supplies used during the manufacturing process. Personnel performing, supervising, or controlling any significant step of the process should be listed in the batch record. Any quality assurance
investigation made during the process (according to 211.192) should also be documented.

To thoroughly control the safety and consistency of a manufacturing process, a quality control program should at least assess:

- starting material (cell or tissue) sourcing and raw materials qualifications,
- characteristics of pre-production cells,
- critical process steps through In-Process Control testing,
- and final product properties through Final Release testing.

5.2 Cell and tissue sourcing

Quality control of the manufacturing process starts with cell and tissue sourcing, also known as “collection.” To prevent the transmission of communicable diseases from the cell donor to the recipient of the therapy product, determining donor eligibility, donor screening, and testing are all required by cGTP regulations (§ 1271.215). This is especially important for allogeneic cell therapy applications. For autologous applications, where the donor and the recipient are the same individuals, donor eligibility determination or donor screenings are not requested (21 CFR 1271.90(a)). However, if manufacturing procedures have the potential to increase the risk of propagating pathogenic agents that may be present in the donor, then the donor should be tested for these pathogens and the results should be documented. Typically, autologous cells and tissues are considered potentially infectious and manipulated as such.

Cross-contamination between cells of different patients is a key safety issue. To address this issue, a proper quality control program begins with specific, documented, and controlled labeling of the specimens harvested from the patient. For privacy issues, a unique patient identifier is assigned to each patient and recorded with his or her name on a log accessible only to the clinical staff. The labeling follows the patient material whenever it changes containers. Forms requiring a witness’s signature or the use of a bar code labeling system can ensure stringent traceability from the initial biopsy to the final product. At the patient bedside, the final product labeling is reconciled with the patient
identity before implantation or injection. Very strict segregation is also required for different batches of cells or tissues being processed simultaneously. This can be achieved through dedication of specific equipment to specific batches as well as by thorough and validated cleaning of shared equipment before each use.

Unless processing occurs in a closed unit at the patient bedside, which is unlikely for applications that involve extensive ex vivo manipulation, collected cells or tissues must be transported to the processing site, usually by shipping. There are many requirements to properly control the shipping of the specimens. Each patient specimen must be packaged in a dedicated, properly labeled, shipping container whose specifications should prevent the contamination or cross-contamination of the material shipped. The containers should be sterile, endotoxin-free, and leak-proof. In addition, the surrounding shipping package should provide environmental conditions (e.g. temperatures ranges) that ensure that the biological characteristics and viability of the material shipped will be properly preserved. Both the shipping containers and the package should comply with the Pressure Differential and Thermal Shock requirements outlined in the Department of Transportation’s Title 49 CFR (§173.196 (a)(6) and (a)(7))24 and IATA regulations, and be validated to maintain their specifications in any shipping environment. Optimally, environmental conditions inside the package should be recorded during the whole shipping process and documented.

5.3 Cell culture

Cell extraction is usually the next step of the manufacturing process. Extraction typically requires the use of enzymes of animal origin, like collagenase or trypsin. These enzymes, as well as any biological raw material of animal or human origin, used in any manufacturing step, should be demonstrated to be free of adventitious agents, including bacteriae and fungi, mycoplasmas, mycobacteriae, and viruses.25

Further requirements apply to biological raw materials. For instance, processes used to remove or inactivate potential infectious contaminants from biological raw materials should be validated. Sourcing, donor screening data (for human sourced-reagents26), and the results of adventitious
agents testing must also be fully documented. Useful recommendations regarding the testing of all materials of animal origin (e.g. enzymes, adhesion factors, albumin, growth factors, etc.) can be found in Refs. 25 and 26. The type of testing necessary depends on the animal species from which the materials are sourced. Because of the risk of Bovine Spongiform Encephalopathy transmission, reagents of bovine origin are of special concern. Interacting with regulatory agencies early during the development of the manufacturing process is valuable for identifying questionable reagents and either finding alternative reagents or determining appropriate actions to minimize the safety risk associated with those reagents.

The composition and purity of raw materials of non-biological origin should also be thoroughly documented. It is very advantageous to use materials that are already licensed for human use.

Whether extracted cells are subsequently expanded, banked (stored frozen) or immediately used for further production, it is required that pre-production cells (or bulk cells) be characterized (see 21 CFR 610 for relevant required tests). The objective of this characterization is to verify that the cells are suitable for further production. Proper bulk cell characteristics increase the likelihood of manufacturing a final product with adequate biological properties.

Generally, bulk-cell identity, purity, viability, and microbial safety must be assessed. However, the consequences that a drift in the bulk-cell characteristics might have on the properties of the final product must be evaluated. Results from this risk evaluation are necessary to determine the extent of necessary cell characterization and will dictate whether additional testing might be necessary (e.g. tumorigenicity, etc.). Therefore, tests should be established during the development of the product to help identify which cell characteristics are necessary for the production of an adequate product. Based upon historical data, a set of acceptance criteria can then be determined for pre-production cells. Those criteria are subsequently used as a means to control the quality of these cells.

In many instances, extracted and expanded cells are banked. Most often, banking cells for cell therapy applications involves establishing a Master Bank. Working Banks are then created by expanding cells from the Master Bank and then using them to manufacture the product. For autologous applications, however, the number of cells can be limited and it
may not be possible to create a Master Bank. Regardless of the cell-banking strategy, viability and phenotypic stability of the cells during cryostorage should be established.

Assessing microbial safety of pre-production cells entails testing for sterility (absence of bacteriae, yeast and fungi per USP〈71〉32 or 21 CFR 610.12), endotoxin amount (per USP〈85〉),33 and mycoplasma (per FDA’s “Points to Consider in the Characterization of Cell Lines used to Produce Biologicals”).34 To prevent spreading microbial contaminations to other patient cells, sterility and mycoplasma should be tested before the cells are transferred to liquid nitrogen storage containers for cryopreservation. In liquid nitrogen storage, viruses (Hepatitis viruses) can spread from one sample to another.35,36 Therefore, vapor-phase nitrogen storage is preferred. Note that testing for the presence of specific human viruses, including CMV, HIV-1 and -2, HTLV-1 and -2, EBV, B19, HBV, and HCV, is not required for autologous cells banks. However, for cells that have been exposed to bovine or porcine components (e.g. serum, serum components, trypsin), it is appropriate to test for bovine and/or porcine adventitious agents.37

5.4 In-process testing

Quality control tests should be performed along each critical step of the manufacturing process through In-Process Control testing. During the manufacturing of cell-based products, microorganisms and impurities can be introduced from multiple sources, including components of biological and non-biological source, poorly controlled process steps, or failures in aseptic processing techniques. To thoroughly control the manufacturing-process quality, each step of the process must be assessed to identify any potential routes of microbial contamination or impurities. This type of risk assessment should take into consideration different parameters, such as:

- materials sources,
- level of manipulation (e.g. multiple or few manual processes, automated processes, no manipulation), and the processing path (e.g. open, partially closed, closed, sealed),
• cell culture or incubation duration (longer times are riskier),
• cell culture vessel (e.g. open (flask, tube, dish), partially closed (sterile collection bag), or closed (bioreactor) system).

Results from this risk assessment should be used to determine critical time points for testing, the most appropriate samples to harvest, and the tests to be performed at these times. For example, in-process sterility testing might be performed during an extended period of culture or after manufacturing steps involving manual processing.

Similarly, it is advisable to test an intermediary-step product for potency (i.e. measuring the product characteristics and biological activity that contribute to its function) or identity immediately after a manufacturing step that is designed to (or that could) modify those parameters, e.g. cell expansion, differentiation, gene modification, etc.

Typically, in-process materials should be tested for sterility, endotoxin, identity, strength (concentration and potency), quality, and purity, as appropriate. The test methods used for in-process control are at the discretion of the manufacturer. However, regulatory authorities will review the testing procedures, the justification of their choice, their limits of acceptance, and the rationale for these limits. Most importantly, valid in-process specifications for such characteristics should be consistent with the final product specifications. They should be derived from previous process averages and variability estimates, where possible, and determined by the application of suitable statistical procedures, where appropriate. In-process control tests are the responsibility of the quality control team and their results are part of the batch records. The quality assurance team will audit them as part of the final-product release.8

As specified in 21 CFR 211.10, written procedures (SOPs) detailing each manufacturing step must be established and followed carefully at all times. They should include the description of the in-process controls, the tests or examinations to be conducted, and the appropriate samples of in-process materials to be tested for each batch. In-process control procedures are useful in monitoring the output and validating the performance of the manufacturing steps that may be responsible for causing variability in the characteristics of in-process materials and the final product. Performing such tests during the manufacturing process allows
for the early identification and rejection of compromised batches of products downstream and immediate investigation of the integrity of the manufacturing step.

5.5 Final release testing

At the end of the manufacturing process, according to 21 CFR 211.165, each batch of product to be released should be appropriately tested to determine satisfactory conformance to final specifications, including identity, strength (or potency), purity, and freedom from contaminating microorganisms. For aseptic products, sterility and absence of endotoxins are essential qualities (21 CFR 211.167). For products whose biological properties depend on the presence of specific viable cells, testing for viability and purity of the cell population is also critical. Sampling and testing plans for final release should be established and described in written procedures that should include the method of sampling and the number of units per batch that will be tested. For each test, or each specification, the acceptable quality level must be set based on a well-documented record of developmental data. The selection of these levels, also called acceptance criteria, is very important since they will be used in making the decision to accept or reject batches of final products. Typically, acceptance criteria are revised and refined as a historical data record is established, however, they cannot be changed to justify the acceptance of a batch already produced (e.g. during a clinical trial).

The quality control team ensures that batches of products meet each acceptance criteria necessary for their approval and release. The accuracy, sensitivity, specificity, and reproducibility of the test methods employed must be established and documented. Such validation and documentation must be in accordance with 21 CFR 211.194 (a)(2). For cell- and tissue-based therapy products, the tests necessary for final release are listed in Table 4. Note that cell therapy products are exempt from the General Safety Test requirement (per 21 CFR 610.11).

Significant challenges are associated with the final release of cell- and tissue-based products. One of the challenges for small-sized autologous cell therapy batches is that the sampling size might be different from those specified in the regulations, which were originally developed for
large-sized batches of pharmaceutical drugs. Acceptable sampling size and strategy will have to be negotiated on a case-by-case basis with the appropriate regulatory authorities. Other challenges stem from the fact that most cell-based products are manufactured using aseptic manipulations because they cannot undergo sterile filtration or terminal sterilization. This makes the assessment of sterility, absence of endotoxin, and freedom from mycoplasma paramount. However, many cell-based products cannot be cryopreserved or otherwise stored without affecting viability and potency. Thus, their shelf life is shorter than the standard test read out times. Test durations for sterility and mycoplasma testing (per CFR 610.12) have been standardized as 14 and 28 days, respectively.29,34

Until recently, in the absence of rapid and effective microbiological testing that avoided administrating the final product before the final sterility test results became available, the FDA recommended a program tailored specifically for these products.38 This program combined standard microbiological testing (sterility, endotoxin, and mycoplasma) initiated a few days (48 to 72 hours) before the final release of the products with a Gram staining performed at the time of shipping or implantation. Another microbiological sample was also harvested at the time the final product was shipped. No-growth results from the 48- to 72-hour sterility test and the negative Gram stain were used for final release criteria. During the last

<table>
<thead>
<tr>
<th>Required Test</th>
<th>Relevant Biologics Standard</th>
<th>Test Method</th>
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<tr>
<td>Potency</td>
<td>21 CFR 610.10</td>
<td>Not specified</td>
</tr>
<tr>
<td>Sterility</td>
<td>21 CFR 610.12</td>
<td>Specified</td>
</tr>
<tr>
<td>Purity (pyrogenicity)</td>
<td>21 CFR 610.13</td>
<td>Specified</td>
</tr>
<tr>
<td>Identity</td>
<td>21 CFR 610.14</td>
<td>Not specified</td>
</tr>
<tr>
<td>Constituent materials</td>
<td>21 CFR 610.15</td>
<td>Not specified</td>
</tr>
<tr>
<td>Mycoplasma*</td>
<td>21 CFR 610.30</td>
<td>Specified</td>
</tr>
<tr>
<td>Communicable diseases</td>
<td>21 CFR 610.40</td>
<td>Not specified</td>
</tr>
<tr>
<td>Viability</td>
<td></td>
<td>Not specified</td>
</tr>
<tr>
<td>Phenotype</td>
<td></td>
<td>Not specified</td>
</tr>
</tbody>
</table>

*Only required for cells that are cultured.
couple of years, *alternate microbiological tests* have been developed and are now available for cell therapy products with a limited shelf life. Rapid microbiological methods are designed to provide performances equivalent to the sterility testing methods described in 21 CFR 610.12, while providing results in significantly less time. Those alternative tests are still not standardized and must be validated, as required for most methods of final product testing under 21 CFR 211.165(e) and 211.94(a)(2). The principles of validation specific to rapid growth-based microbiological methods are described in Guidance for Industry Validation of Growth-Based Rapid Microbiological Methods for Sterility Testing of Cellular and Gene Therapy Products.  

Recently, rapid mycoplasma detection techniques using Polymerase Chain Reaction (PCR) have been developed as well. Prior to licensing, data demonstrating that the PCR test selected has a sensitivity and specificity similar or greater than the standard test must be provided.

Guaranteeing final product safety by assessing the *potency* of the final product constitutes another huge challenge for autologous biological products. Potency of an autologous product often varies from one patient to another. Live cell- and tissue-based therapies frequently rely on the activity of cell populations that are not 100% pure or on the combined activities of several cell populations that can have complex mechanisms of action. The characteristics of autologous cell therapy products that make the development of potency tests difficult are described in Table 5. Historical developmental data, relevant preclinical information, and early clinical study results can be used to determine which product attributes are most relevant for measuring potency.

For products that contain more than one active ingredient, potency measurements should be designed to assess the biological activity of all active ingredients. The potential for interference and synergy between active ingredients should be considered. A collection of assays (biological and/or analytical assays) can be developed for products with complex mechanisms of action or presenting multiple biological activities. At least one quantitative assay must be included in the combination. Analytical assays can evaluate immunochemical, biomechanical, and molecular attributes of the products. Their data should be correlated with a relevant product-specific biological activity.
Table 5.  Autologous Cell- and Tissue-Based Product Characteristics that Make the Development of Potency Test Difficult.

<table>
<thead>
<tr>
<th>Challenges to Potency Assay Development</th>
<th>Examples</th>
</tr>
</thead>
</table>
| Inherent variability of starting materials | • Donor variability  
• Cell line heterogeneity |
| Limited lot size and limited material for testing | • Single-dose therapy using cells suspended in a small volume  
• One single lot per patient |
| Limited stability | • Viability of cellular products |
| Lack of appropriate reference standards | • Autologous cellular materials |
| Multiple active ingredients | • Multiple cell lines combined in final product  
• Heterogeneous mixtures of immune-modulating cells |
| Potential for interference or synergy between active ingredients | • Multiple cell types in cell preparation |
| Complex mechanism of action(s) | • Multiple characteristics required for appropriate functions (e.g. biomechanical resistance and anti-thrombogenic properties for an autologous tissue-engineered blood vessel)  
• Multiple potential effector functions of cells |
| In vivo fate of product | • Migration from site of administration  
• Cellular differentiation  
• Remodeling of tissue-engineered construct  
• Invasion by recipient own cells  
• Immune response to components introduced during manufacturing process |

To market a biological product, a validated potency assay with defined acceptance criteria must be described in the BLA (21 CFR 211.165(e)). The acceptance criteria, intended for subsequent final release testing, should be based on knowledge gained through manufacturing experience and data collected during all phases of product development and clinical investigation. Reference material used as controls for the assays should be available and can include well-characterized clinical lots. Several resources are available for analytical method validations.41–43

5.6 Shipping the final product

After final release, biological products are either stored or shipped to the patient bedside. Requirements similar to the ones pertaining to biopsy shipping, labeling and packaging, apply to the final product shipping. Supplementary labeling (e.g. “For Autologous Use Only”, “Not evaluated for infectious diseases”, “Warning: advise recipient of communicable disease risk”, “Biohazard”, etc.) might also be required.44 In addition, under 21 CFR 211.166, an adequate number of batches must be tested to determine the product stability under specific storage and shipping conditions. The results of such stability testing are used in determining appropriate storage and shipping conditions as well as in establishing the product shelf life. Stability of such products should be assessed in the worst shipping conditions possible (duration, temperature) in the same container system in which the product will be marketed. For further information, refer to ICH 5C, Q1A(R2), and Q1E.45–47 Typically, living autologous cell- and tissue-based therapy products will have a short shelf life.

6. Conclusion

Cell- and tissue-based therapy applications, especially those requiring extensive, and sometimes lengthy, ex vivo cell manipulation present an additional challenge for both regulatory authorities and the companies developing them. The challenge lies in the necessity to strike a balance between the quality control official’s need to ensure product safety and the company’s ability to comply. More simply put, the extent and number of
the quality control steps, which tend to multiply with the complexity and length of the manufacturing process, need to guarantee patient safety, but should not prevent a much-needed treatment from becoming clinically available. Although many autologous somatic cell therapy applications have been developed so far, only a few are commercially available. In the United States, only two autologous cell therapy products requiring extensive ex vivo manipulation and regulated through the BLA pathway have yet reached the market. Carticel®, produced by Genzyme Tissue Repair, became available in August 1997 and Provenge®, from Dendreon Corporation, in April 2010. Over the last decade, a significant discrepancy has existed between the rapid development of cell- and tissue-based therapies and their slow access to the market. This discrepancy has incited various companies to develop and test new applications outside of the United States. Hopefully, a more progressive political context will allow the FDA to accelerate the rate of approval for such therapies so that more US patients can safely benefit from them.

Acknowledgments

The authors thank Marissa Peck and Corey Iyican for their help in revising and editing this chapter.

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1. Introduction

Interactions with the Food and Drug Administration (FDA) or other regulatory agencies typically conjure images of a bureaucratic quagmire at best, or an adversarial fight at worst. In all fairness, these regulatory bodies are tasked with an incredibly challenging situation: trying to balance the mutually exclusive demands for a “least burdensome process” from industry with a “zero risk” expectation from the patients and overly eager litigators behind them. Industry is quick to criticize the FDA for its lengthy approach to review and approval, and the lay press is quick to chastise the agency over rare clinical debacles such as those associated with Vioxx (Merck) or TBN 1412 (TeGenero). Few people, however, laud these same regulatory agencies for presiding over nearly 50 years of medical innovations in the modern era of drug and device regulation. Indeed, in the post-thalidomide era, the average life expectancy has increased from roughly 70 years in 1962 to nearly 80 years in 2003. In effect, we
can thank the FDA for safely adding more than two hours to each 24-hour day that we live.

While there have been some dramatic failures of drugs and devices, none in the modern era of regulation have approached the scope of the thalidomide tragedy. When viewed in light of the billions of devices and drug doses that have been safely administered, the overall performance of the worldwide regulatory bodies is commendable. Safely managing the transition of medical innovations from bench-top research to clinical use has driven this improvement in longevity and quality of life. This is even more remarkable in light of the funding disparity between the regulatory agencies and the scientific communities. Fueled by an annual budget of approximately US$30 billion from the National Institutes of Health (NIH) and billions more from industry, medical innovation has leapt forward at unfathomable rates. The FDA is understandably hard pressed to keep pace with this innovation on an annual budget of just over US$2 billion. As late as the early 1960s, considered the dawn of the modern era of drug and device regulation, the structure of DNA was still unknown, catheter-based cardiovascular interventions were essentially non-existent, and drug therapies for cancer and cardiovascular disease were in their infancies. Thus, a realistic and sympathetic approach should be sustained when setting expectations for regulatory agencies to keep pace with innovations such as gene therapies or Regenerative Medicine.

While it is appropriate to acknowledge the unenviable tasks assigned to the FDA and other regulatory agencies, it is clear that there are unnecessary burdens in each system, particularly with respect to new therapies, in particular Regenerative Medicine and cell-based therapeutics. Indeed, there are dramatic regional disparities in the data required to support submission, the tolerance of risk, and the subsequent time required to receive the study approvals. Additionally, there are significant differences in costs associated with clinical studies and access to patient populations. In this chapter we will explore some of these regional differences and provide insights into more efficient management of the regulatory processes, irrespective of location. As evidenced by the dramatic increase in the number of clinical trials performed outside countries like the US, France, Germany and the UK, it is clear that developing a cohesive global clinical trials strategy has become a critical priority for a burgeoning Regenerative Medicine company.
Specifically, in this chapter we will address:

- Key regulatory considerations for each clinical trial phase
- Strategies that target certain geographical regions for specific developmental advantages
- Use of a Clinical Research Organization (CRO)
- Universal regulatory considerations for cell-based therapeutics

2. Regulatory Challenges at Each Phase of Clinical Development

2.1 Clinical trials design considerations

While it may be obvious, it should be stated at the outset that a well thought out clinical trials strategy is perhaps the most important element to consider in the process of transitioning to human use. It is difficult to make sweeping recommendations relating to protocol design, as each trial will be driven by its own particular requirements. It is worth emphasizing, however, that the appropriate selection of endpoints can make the difference between approval and rejection of the protocol from a regulatory perspective, and more broadly, can make the difference between perceived success and perceived failure of the study. While the perception of the latter point is beyond the scope of this chapter, one should realize that setting aggressive endpoints can result in adverse interpretations from the Data Safety Monitoring Board (DSMB) or the regulatory agencies once the data is reviewed. For example, in our own Phase I/II trials involving hemodialysis access, a cell-based vascular graft was used in a subset of “worst case scenario” patients. This type of protocol is quite common with radically new technologies. Data relating to the standard of care, typically reported across a much broader patient population, predicts a life expectancy of nearly six years. So, in the context of the standard of care, it is tempting to set Phase II endpoints at 12 or even 18 months, as is typically done with the standard patient population. However, with the short life expectancy unique to our subset of patients (one to two years), we would have been unlikely to meet these endpoints. This same argument can apply to Phase I studies in Regenerative
Medicine, as often there are secondary endpoints that relate to longer-term efficacy. Regenerative Medicine trials rely upon complex biological mechanisms, and it is difficult to accurately predict the outcomes and the most appropriate endpoints. To minimize the likelihood of studies being suspended due to outcomes that are unexpected or difficult to interpret, one strategy we have employed successfully is defining clear failure criteria and continuation criteria in the original protocol. By negotiating failure and continuation criteria upfront, there is less pressure on the DSMB or regulatory agencies to suspend a study that has an unexpected result.

While there is no question that the regulatory challenges facing the clinical development of cell-based therapeutics are onerous, there are many shortcuts that can, and should, be made to streamline the overall approval process. It is important to understand that philosophically, the FDA is completely risk averse and depends largely upon a strategy of exhaustive data collection to demonstrate a thorough review. While sponsors tend to shy away from direct confrontation with the FDA or similarly established regulatory agencies, these agencies, particularly in Europe, are actually quite responsive to rational arguments against unnecessary data collection. So, while the guidance documents may encourage detailed, standardized testing pulled from every conceivable precedent, many of these tests can be eliminated from lot release criteria (or at a minimum be done a single time as a validation study) if there is supporting rationale. Moreover, as detailed below, some experimental “requirements” can be delayed until Phase II or even Phase III studies are to be performed.

The classic lines defining Phase I, II, and III trials are often blurred in Regenerative Medicine. While some simple cell-based injections (myocardial regeneration, for example) may require a classic Phase I dosing study, even these initial safety studies have efficacy endpoints. Thus, we tend to call our initial studies Phase I/II studies, which are clearly distinct from controlled Phase III studies. So while the study phases may be defined differently for specific applications, the recommendations below may accelerate clinical studies. These strategies, however, should be considered as a part of a detailed risk analysis to ensure that these “shortcuts” do not appreciably compromise patient safety.
2.2 Phase I/II clinical testing

Most companies do not fully appreciate or take advantage of the relaxed manufacturing requirements for Phase I trials. While the FDA does not encourage “garage” manufacturing during pre-IND (Investigational New Drug) meetings, the guidance documents very clearly establish that there is no requirement for Good Manufacturing Principles (GMP)/Good Tissue Practices (GTP) manufacturing at this stage of clinical development. This is not the case for Europe, as GMPs are required for products manufactured for human clinical trials. However, even in Europe, the regulatory bodies tend to offer some leeway in co-developing a company’s various GMP processes early on, especially if it is an SME (small and medium-sized enterprise) and/or the company does not yet have a product on the market. Given the fact that early stage money is extraordinarily expensive (that is, early valuations are low, making early stage expenditures disproportionately “expensive” from a capitalization perspective), it makes little sense to implement overly extensive quality assurance/quality control (QA/QC) procedures for Phase I trials. For most Phase I cell-based therapies trials, the key manufacturing elements that affect patient safety (functionality, uniformity, sterility, avoidance of cross-contamination, etc.) can be consistently performed in any reasonably organized university lab as long as the appropriate release criteria are employed. Relatively basic documentation of the key manufacturing steps usually suffices for initial studies. These basic systems can then be filled in as the study progresses with more detailed procedures and hardware. Indeed, our own approach to Phase I clinical trials was to establish GMP-level manufacturing controls for the key, high-risk steps in the manufacturing process, as well as for those repeated frequently, such as change of cell culture medium. This more focused GMP effort significantly augments product safety without unnecessary early stage expenditures. Additional safeguards, such as stringent functional release criteria, conservative enrollment rates, and long post-treatment surveillance periods, were employed. We felt that these safeguards probably did more for overall patient safety in Phase I trials than the more costly efforts later required to bring the full production process up to GMP standards. Our experience with eventually building a GMP-compliant manufacturing facility was that
80% of the cost was applied to the final 20% of the facility. This final 20% focused on relatively obscure details that had little impact on the overall risk analysis for our small, early-stage trials. This recommendation of a streamlined QA/QC process should not be confused with a poorly defined manufacturing process. It should be noted that even minor changes to the manufacturing process itself can result in the necessity to repeat Phase I studies (or similarly perform a validation study to demonstrate equivalence between the processes). Therefore an important strategy in Phase I trials is to therefore to make every attempt to finalize and document the details of the manufacturing process. Similarly, in vitro functional assays should be developed that can be used in lieu of clinical validation studies to demonstrate equivalence in the likely event that process changes are made as the technology evolves through Phase II and Phase III studies.

Some aspects of manufacturing controls or lot release criteria are deemphasized in regions with emerging clinical programs. It is tempting to take advantage of these relaxed requirements in a globalized clinical trials strategy. However, since FDA or Western European reviewers will analyze the enrollment and manufacturing safeguards in place for earlier studies before accepting human data, it is essential to design the clinical study as if it were being conducted in Western Europe or the US. The principal advantage to a globalized clinical trials approach is primarily associated with decreased study costs and a quicker process for receiving study approval, as discussed later in this chapter.

A final point worthy of mention for Phase I studies is the use of Clinical Research Organizations (CROs). A CRO is a third-party company that provides a variety of services for clinical trials sponsors, including clinical trials management, medical and safety monitoring, and data management and analysis. The common perception among Regenerative Medicine companies is that regulators require the use of CROs. These entities may seem omnipresent in all stages of the clinical trials process, but careful review of the guidance documents in most countries reveals that while outside, independent monitoring is encouraged, it is not required (a DSMB is required for each trial, in part to provide independent consideration for major trial decisions). While the CROs do provide convenience and some guidance, the use of a CRO ultimately becomes an important issue when cost is factored in. Even in relatively inexpensive
regions, CROs seem to have uniformly elevated their fees to American levels. Most clinical cost estimates we have seen show that more than 50% of the overall clinical costs for all studies are associated with regulatory submissions, legal representation, and clinical monitoring performed by CROs. Moreover, budget overruns associated with trials outside the US and Western Europe are almost always associated with the CRO and not the clinical study itself. While additional discussion on this topic can be found later in the chapter, overall, we recommend careful cost analysis and vigilant contractual navigation when partnering with a CRO.

2.3 Phase II/III clinical testing

Transitioning to Phase II/III trials brings with it the responsibility of full GMP and GTP manufacturing compliance and all of the associated costs. While the requirements for GMP manufacturing are discussed elsewhere in this book, there are two key elements to Phase II/III clinical trials that should be carefully considered: endpoints that demonstrate geographic distribution and cost-effectiveness.

2.3.1 Geographic distribution

There is clear pressure from investors to gain early access to the large US and EU markets, regions that are also the most likely to support the price premiums required from cell-based therapeutics. While this makes sense for classic drug and device business models, the model is less relevant for cell-based therapies, where the overall sale volumes have been low. Even the most successful commercial products, such as Organogenesis’ Apligraf® or Advanced BioHealing’s Dermagraft®, sell to a very small fraction of the wound healing population. There are no cell-based therapies that sell in sufficient volumes to claim a penetration rate of more than a few percentage points. Said another way, even these icons of industrial success, which are both clinically efficacious and profitable, sell little more than a few tens of thousands of units per year. Moreover, current manufacturing capacities by most Regenerative Medicine companies cannot service significant penetration in these largest markets. Thus, one can make the argument that a slow-growth business model, even in a smaller...
market, could provide the basis for a successful outcome. Our own clinical trials strategy is to continue with Phase II/III clinical studies in Eastern Europe and South America to lower the overall cost and expand to a single Western EU country to facilitate eventual commercialization in Europe. For our clinical indication, which addresses an extremely large patient population, even modest penetration into a single country like Germany would represent a major commercial success for a cell-based therapy and would likely exceed our current manufacturing capacity. Thus, the typical strategy of concurrent regulatory and commercial activities in both the US and Western Europe should be carefully evaluated.

2.3.2 Cost-effectiveness

While clearly outside the scope of regulatory considerations, clinical trials design should also be mindful of the increasing pressure to demonstrate cost-effectiveness. While Medicare policy does not acknowledge the requirement for demonstrating cost-effectiveness, reimbursement for cell-based therapeutics will absolutely require convincing data on cost-effectiveness. Treatments for life- or limb-threatening diseases or orphan diseases, where few or no clinical options exist (certain cancer vaccines, for example), may be immune. In general, however, our industry will be required to demonstrate that the cost of our treatments can compete with the standard of care or that higher costs are justified by an increased life span and/or improved quality of life. This realization is critical for clinical trials design, as a rigorous sponsor will tailor their trials to both overcome short-term commercialization hurdles and demonstrate cost-effectiveness in the long-term. For example, in the United States, the Centers for Medicare and Medicaid Services (CMS) is the institution that selects healthcare products that are reimbursed by the government; thus, demonstrating cost-effectiveness to CMS is as significant a task as earning a commercialization permit from the FDA.

3. Regional Considerations for Clinical Trials

Historically, smaller medical device companies and Regenerative Medicine companies have focused on early clinical trial approvals in the
US and Europe. These approvals are seen as key milestones, as they are associated with access to larger markets and can lead to a subsequent increase in valuation. While this is more relevant for Phase III trials, Phase I and II trials, particularly for an SME in the field of Regenerative Medicine, should be driven more by cost and speed considerations. The key valuation inflection point for fledgling Regenerative Medicine companies with a limited funding horizon is first-in-man studies. As long as Good Clinical Practices are followed (suggesting the data would ultimately be admissible as a part of later stage EU or US regulatory applications), it would seem appropriate to avoid spending expensive early-stage capital by choosing a region with lower costs and more rapid approvals. Indeed, the appeal of lower cost and faster study approval has driven a significant globalization of clinical trials. That is, there has been a shift towards performing early-stage clinical trials in such countries such as Russia, Brazil, China, and India. Interestingly, the US provides an unparalleled entrepreneurial and scientific environment for innovation, yet the US’s complete aversion to clinical trials risks and associated litigation problems are driving access to cutting edge clinical treatments out of the country.

In deciding where clinical trials should be performed, a balance must be struck between the overall cost, speed of study approval, access to an appropriate patient population, quality of care, and the perceived quality of the data produced (e.g. does clinical data from India impart the perception of sufficient quality to a regulatory agency in Germany?). Trumping all of these questions, however, should be the selection of a Principal Investigator (PI) and a CRO. Ultimately, the value of the clinical study is directly related to the quality of the data collected, and it is the PI and the CRO that will create, collect, and manage this data. Table 1 summarizes by region the difficulty ranking of the various regulatory hurdles discussed in this section.

3.1 United States

The United States market is a double-edged sword for Regenerative Medicine. The US offers a single, centralized regulatory agency for both clinical trials and commercialization, with a now well-established regulatory framework for cell-based therapeutics. A very clear set of guidance documents, with “Guidance for Human Somatic Cell Therapy and Gene
Table 1. Ranking by Region of Difficulty of Completing Various Regulatory Processes for Cell-Based Therapeutics.

<table>
<thead>
<tr>
<th>Region</th>
<th>Clinical Trial Approval Process</th>
<th>Clinical Trial Relative Cost to Complete Clinical Trial</th>
<th>Perceived Value of Clinical Data</th>
<th>Commercialization Approval Process</th>
<th>Reimbursement Process</th>
</tr>
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<tbody>
<tr>
<td>United States</td>
<td>Difficult</td>
<td>High</td>
<td>High</td>
<td>Difficult</td>
<td>Fair</td>
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<tr>
<td>European Union</td>
<td>Moderate</td>
<td>Moderate to high</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate to difficult</td>
</tr>
<tr>
<td>South America</td>
<td>Low to moderate</td>
<td>Low to moderate</td>
<td>Low to moderate</td>
<td>Moderate</td>
<td>Fair to difficult</td>
</tr>
<tr>
<td>East Asia</td>
<td>Low to moderate</td>
<td>Low to moderate</td>
<td>Low to moderate</td>
<td>Moderate</td>
<td>Fair to difficult</td>
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</table>
Therapy” and “Sterile Drug Product Produced by Aseptic Processing” at its core, define this framework.\textsuperscript{6,7} In addition, the US offers both a high rate of reimbursement and a huge potential market for sales, with a population of roughly 300 million people and a large fraction of elderly inhabitants.\textsuperscript{8} However, due to a highly litigious society and huge political pressures to avoid scandal, the FDA is shifting more and more to a policy of complete risk avoidance. Thus, the FDA’s approval processes are well established and predictable, but they are inflexible and require large volumes of experimental data. While the FDA may attempt to evaluate products on a case-by-case basis, the truth is that the regulations are largely fixed and the agency typically errs on the side of extreme caution. Indeed, the US system seems to encourage the rise of globalized clinical trials for first-in-man studies. Even in the medical device markets, companies now tend to seek commercialization in Europe first, and then later, if at all, in the US. As an example, there are approximately 15 repair devices for abdominal aortic aneurysms (AAAs) CE-marked for sales in the EU, but only about five available for use in the US.

\section*{3.2 \textit{European Union}}

The European regulatory framework is advantageous due to its unique combination of regional and centralized controls. Clinical trials are approved and performed under regional authority, taking advantage of cost/approval advantages in countries such as Poland. Commercialization to all EU member states is then controlled by a single centralized entity, the European Medicines Agency (EMA), following the framework set up by the Advanced Therapy Medicinal Products (ATMP) directive and governed by expert committees, such as the Committee for Human Medicinal Products.\textsuperscript{9} This myriad of regulations can be difficult to navigate. For example, in Germany, a company must apply for a clinical trials approval through one state agency, approval of its GMP processes through another, and then a manufacturing permit through the EMA. In France, a company producing cell-based therapeutics must be registered as a national tissue bank, requiring the manufacturer to set up a facility in France. A flowchart summarizing the regulatory process in Europe is presented in Fig. 1. While the regional bureaucracies can create a complicated process, overall, the
Fig. 1. Regulatory pathway for cell-based therapy clinical trials and marketing authorization in the European Union. Note that this pathway refers to advanced therapy medicinal products (ATMPs) placed on the market in Europe after Dec 31, 2008 (and all ATMPs on the market in Europe after Dec 31, 2012). This pathway is not applicable for therapies designated as medicinal devices. Also, this pathway does not include potential post-marketing Phase IV clinical trials that may be requested by the EMA.
access to initial clinical trials and then a large commercial market (over 500 million) can be significantly more efficient and less costly than the US pathway.

3.3 South America

Unlike Europe and US, there is no centralized regulatory body in South America. The advantage to running studies in South America is that these regulatory bodies are significantly more accepting of risk and less litigious than the US or even Western Europe. Thus, South America’s various approval processes, especially for clinical trials, usually are completed much faster than the US and EU. In our experience, it took approximately six months to obtain approval to perform a Phase I trial in Argentina, versus the two plus years it has taken in Germany and three plus in the US. However, this lack of a rigid system can also be a detriment, as the various regulatory systems do not necessarily correspond with one another. Thus, the pathway to approval for clinical trials or commercialization in Argentina will not be the same as Brazil or Ecuador. Finally, reimbursement rates in these countries is much lower than in the US or Western Europe. As such, it has been our experience that it is far more attractive to perform clinical trials in these regions, but to hold off on commercialization.

4. Use of a Clinical Research Organization

The primary driver for conducting clinical studies in emerging countries like Brazil, Russia, or India is cost. While many sponsors are hopeful that these countries represent significantly accelerated access to human use, the actual review processes are typically more thorough and take longer than some may think. When one factors in the additional work of replicating regulatory efforts in Western Europe or the US, the overall time benefit is marginal. So, while there may be some near term advantages in rapid initial access to human trials (Phase I/II), the long-term benefits relate to cost savings. Somewhat surprisingly, the costs of running a clinical study in South America or Eastern Europe can be dominated by the fees paid to a CRO. Careful selection of the CRO and the responsibilities
they will be given is an excellent way to significantly reduce the overall costs associated with the regulatory approval process and the clinical study itself. There are two basic drivers that force the use of a CRO. The first is a general requirement in most countries for legal representation to submit regulatory documents, import medical products, or conduct clinical studies. The second is a general requirement to have a clinical monitor to check source data. While established CROs often perform or manage these roles, there are a variety of other options, detailed below.

Given the uniqueness and complexity of running Regenerative Medicine studies, in our experience, most CROs are ill-equipped to handle trials outside the scope of the classic drug or device. Even those that promote themselves as experts in Regenerative Medicine often have little real expertise in the regulatory, clinical, or logistical requirements of running a study that is based upon the use of a living product. While this knowledge gap is closing as more and more clinical studies are performed in this field, it is safe to say that the sponsor should expect to play a direct and engaged role in the day-to-day management of the clinical trial. Indeed, we have hired our own local employees to help accelerate and manage our regulatory and clinical activities performed outside the US. In most instances, these same local employees, under the tutelage and management of the sponsor’s clinical trials manager, can perform all of the roles normally performed by a CRO at a fraction of the cost. It is important to note that this includes data monitoring. There is generally no requirement to use a CRO or even an independent monitor to perform these tasks. However, the risk of inadvertent mistakes that can be perpetuated when the sponsor is directly responsible for both study design and data monitoring should be evaluated and balanced against the cost savings associated with this strategy. Similarly, the potential for a regulator’s negative perception of an “internal” biased monitor must be evaluated.

CROs can also provide localized legal representation. As an alternative to large global CROs, there is a growing number of smaller contractors that perform this role. We have used entities such as MARES Ltd. in Western Europe and Access Medical Research in South America and Poland with success. This provides a low-cost alternative to a full-time local or regional CRO, but puts significantly more responsibility and workload on the sponsor’s clinical and regulatory teams. On one hand,
this may result in additional direct costs and delay for the sponsor; on the other hand, it will reduce indirect costs paid to a CRO or similar third party company, allow the Sponsor more control over timeline and expenses, and develop clinical expertise in-house that can be used in future trials. Given the relative inexperience of CROs within Regenerative Medicine, we have found this to be an appropriate trade-off.

5. Universal Regulatory Considerations for Cell-Based Therapeutics

Tissue Engineering and cell-based therapies have been hailed as the third pillar of future medicine, alongside devices and drugs. While the field as a whole has tremendous support in academia and has reached important milestones over the last decade, translation to widespread clinical use will be crippled by the current regulatory framework. It is incumbent upon us all to collectively lobby for rational regulations that are more relevant to Regenerative Medicine and result in an appropriate risk-benefit profile for the patient. The regulatory framework in almost every country with established guidance documents on cell-based therapeutics has been derived from aseptic drug manufacturing requirements. The fundamental problems with this approach are the dramatic disparities in cost per “dose” and lot sizes between pharmaceuticals and cell-based therapeutics. With lot sizes in the hundreds of thousands and cost per dose of a few cents, the drug industry can easily absorb the QA/QC costs associated with extensive manufacturing controls and lot release testing. In cell-based therapeutics, however, even most allogeneic products have lot sizes of a few thousand at most, and costs per dose that are orders of magnitude higher than pharmaceuticals. While companies like Organogenesis and Advanced Biohealing have brought manufacturing costs down to the point of profitability (if one ignores the hundreds of millions of dollars in R&D expenses forgiven by bankruptcy), many companies are burdened with more complex products, smaller lot sizes, difficulties in cell sourcing, etc. While it is not in the FDA’s mandate to establish a regulatory system that promotes profitability for industry, it is their responsibility to enable patients to have access to new therapies. The question, of course,
is what level of risk are we willing to tolerate in order to bring QA/QC costs down?

There is a valid argument to be made that the current environment of complete risk aversion does a disservice to both patients and industry. For example, an immuno-compromised, terminally-ill cancer patient would likely accept the small risk of death from a contamination in order to have access to the potentially life-saving benefit of the treatment. We are required to test for sterility, mycoplasma presence, and endotoxin presence for our tissue-engineered grafts at three separate time points in the manufacturing process. In nine years of production, about half of which was performed under non-GMP certified conditions and the remainder under GMP conditions, we have had one test come back as “inconclusive.” Thus, it would seem that our process controls have more than adequately controlled the risk of contamination and that GMP certification did not significantly improve upon this. Similarly, many hospital laboratories have been producing cultured epithelium for burn patients under non-GMP conditions for years without issue.

Ironically, a variety of cell-based products manufactured and/or implanted with reduced QA/QC requirements are considered the standard of care today. For instance, organ transplants are typically performed “as is,” with limited QC evaluation. In addition, “in vitro surgeries” are common, in which the living tissue of a patient is manipulated (often with surgical methods and instruments) and transformed into a construct that is implanted. Also, as previously stated, hospitals currently produce cultured epithelium for burn victims. Obviously, these products are not subjected to the same manufacturing controls as commercial cell- and tissue-based products. Furthermore, all implantable products are implanted in an operating room environment that does not meet the cleanliness standards required for aseptic manufacturing. We certainly could apply the same QA/QC standards required for cell-based therapies to the products listed above to ensure “zero risk” of contamination and error. The result would be that the already exorbitant cost of interventions would continue to swell and further decrease the portion of patients who could afford the procedure. The truth is that this “zero risk” attitude is already a contributing factor to the steep rise of healthcare costs in the US. Thus, if we want to see the day when complex tissue-engineered organs can be produced
and implanted at reasonable cost, regulatory agencies will have to accept some safety trade-offs to reach an acceptable balance of cost and risk.

Another fundamental problem is that the regulatory agencies demand that the sponsor perform every test that has cumulatively encumbered other applicants. The responsibility then lies with the sponsor to whittle down this all-inclusive list that gives an appropriate level or risk-benefit for the particular patient population. While the regulators have the benefit of knowing all the data from prior applications, industry has not typically shared regulatory or manufacturing data so that we can turn the tables and argue for the least common denominator. In other words, knowing what lot release criteria, for example, are required to demonstrate safety and efficacy for a similar product might have dramatic implications in a successful argument for a similarly streamlined lot release strategy. Despite the secrecy that has historically shrouded the development of medical devices and pharmaceuticals, we believe it is critical to the survival of our industry to begin an active data sharing program with respect to regulatory QA/QC requirements. Whenever possible, the FDA and other agencies should provide the data on which they base their regulatory and guidance policies and lay out their decision-making processes to the public.

References

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